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IMMUNOHISTOCHEMICAL CRITERIA

THE RELATIONSHIP BETWEEN

PROSTATIC INTRA-EPITHELIAL NEOPLASIA

AND PROSTATE CANCER.

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Prostatic Intraepithelial Neoplasia and Prostate Cancer.

AN ORIGINAL THESIS SUBMITTED FOR THE

DEGREE OF DOCTOR OF MEDICINE (MD) TO

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BY

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Prostatic Intraepithelial Neoplasia and Prostate Cancer.

Abstract:

Prostatic Intraepithelial Neoplasia (PIN) is a putatitive precursor of prostate carcinoma (CaP). PIN is defined as an abnormal proliferation where the cellular changes exhibit the cytological changes of cancer, but retain a basal cell layer. Using radical prostatectomy specimens we have studied the relationship between PIN and carcinoma of the prostate.

Method:

This study was divided into two parts:

1) Step sections from 76 consecutive radical prostatectomy specimens were examined prospectively. Using planimetry software the total volume of the gland, the volume of cancer, the volume of benign tissue, and the volume of PIN was calculated. These volumes were compared to serum PSA.

2) The expression of p_{53}, bcl_2, bax_{alpha}, and Ki-67 was measured using semi quantitative immunohistochemistry in 92 hormone naïve specimens as well as specimens from 14 patients who had received androgen ablation. Benign prostate specimens obtained from 19 patients were used as controls. This was carried out retrospectively.

Results:

The mean weight of the prostate gland was 61.3 (range = 26 – 173) g, the mean calculated total volume of the gland was 50.9 (range 19.4 –155.5) mls, the mean volume of cancer was 2.5 (range = 0.09 – 15) mls, the mean volume of PIN was 0.53 (range = 0 - 2.7) mls, and the mean volume of benign tissue in each gland was 47.5 (range = 13.3 – 153.4) mls. The mean value for the PSA= 9.1 (range = 3-45) ng/ml.

The calculated volume of the gland correlated with the weight of the gland ($p<0.0005$, $r=0.9353$). The cancer volume also had an inverse correlation with the benign and the total volume of the gland ($p<001$). The volume of PIN correlated with the total volume of the gland.

Prostate weight, volume and cancer volume independently correlated with serum PSA. The volume of PIN did not correlate with serum PSA. 1 gram of benign tissue correlated with 0.07 ng/ml of serum PSA, and one gram of malignant correlated with 0.8 ng/ml of serum PSA.

The expression of p_{53} increased in a stepwise manner, 0/19 benign, 5/92 PIN, and 9/92 CaP, but the increase was not significant. Bcl_2 was expressed in 75/92 PIN specimens, 27/92 Ca P and 0/19 benign specimens ($p<0.05$). Bax_{alpa} expression was ubiquitous, but staining intensity increased when comparing benign with PIN or CaP ($p<0.05$). Ki-67 expression was absent in benign tissue and expressed in 91/92 PIN specimens, and 92/92 with CaP ($p<0.05$). In the subset of patients treated with androgen ablation areas of cancer were compared with specimens from the hormone naïve group. P_{53} was expressed in 4/14 pts ($p=0.07$), bcl-2 expression occurred in 9/14 ($p<0.05$), bax_{alpa}-staining intensity decreased ($p<0.05$), and the number of cell expressing Ki-67 decreased ($p<0.05$).

Conclusions:

PIN volume correlates directly with gland volume, the volume of cancer correlates inversely with the volume of benign tissue. 1 gram of malignant tissue correlates with eleven times as much serum PSA when compared to benign tissue. The volume of PIN does not have any relationship with serum PSA.

P_{53} expression is infrequent and may not be important in the early genesis of CaP. Bcl_2 expression increases from benign to PIN and decreases in CaP. It is expressed in the luminal cells, and confers relative immortality, resistance to cytotoxics and androgen ablation. Therefore, PIN luminal cells may have the survival advantage required for progression from PIN to CaP, and are relatively resistant to intervention. These cells may also represent the precursors of androgen resistant clones. The increased intensity of bax_{alpha} in CaP, allows it to be amenable to therapeutic intervention. Ki-67 positive cells shift from basal to luminal as one progresses from benign to malignant tissue. This implies increasing cell production in areas of malignant tissue.

Androgen ablation leads to a decrease in Ki-67 staining, an increase in the proportion of specimens expressing bcl_2 and p_{53}, and a decrease in the staining intensity for bax_{alpha}. Therefore LHRH decreases the production of cells, may use p_{53}-mediated pathways for apoptosis and by up regulating the expression of bcl_2 may cause the selection of androgen resistant immortal clones.

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Chapter 1: Introduction

1.1 Embryology and Foetal Pre-Pubertal Histology

The male foetus under the control of the product of the SRY gene from the Y chromosome produces the SRY protein. The SRY protein initiates a developmental cascade which results in the formation of the testis, male genital ducts, and associated glands, the male external genitalia, and the entire constellation of male secondary sex characteristics.

In the third month the indifferent pelvic urethra is transformed. The prostate begins development from the mesenchyme surrounding the urogenital sinus [12]. Its development depends upon dihydrotestosterone, which is produced from foetal testosterone by the enzyme 5-alpha reductase in the urogenital sinus [85]. Epithelial buds invaginate from the posterior urogenital sinus on either side of the verumontanum. Simultaneously the mesonephric (Wolfian) ducts develop into the seminal vesicles, epididymis, vas deferens, and ejaculatory ducts, which are stimulated by foetal testosterone. By 16 weeks of gestation the basic structure of the prostate is complete.

The glandular buds, in the foetal prostate, develop as solid outgrowth of cells. Small lumina develop lined by cuboidal or columnar epithelium [133]. These glands are simple tubular structures without significant branching, and are

lined by multiple layers of immature cells with round nuclei and scant cytoplasm.

1.2 Gross Anatomy of the prostate.

The non-pathological prostate in the post pubescent male weighs 12-20g and is the shape of an inverted cone. The size of the prostate enlarges with age, in those that are over 40, the average prostate is the size of a walnut and measures approximately 4.0-4.5cm (transverse diameter), 2.5-3.0 cm (a-p diameter), and 3.0 – 4.0cm (cephal-caudal diameter).

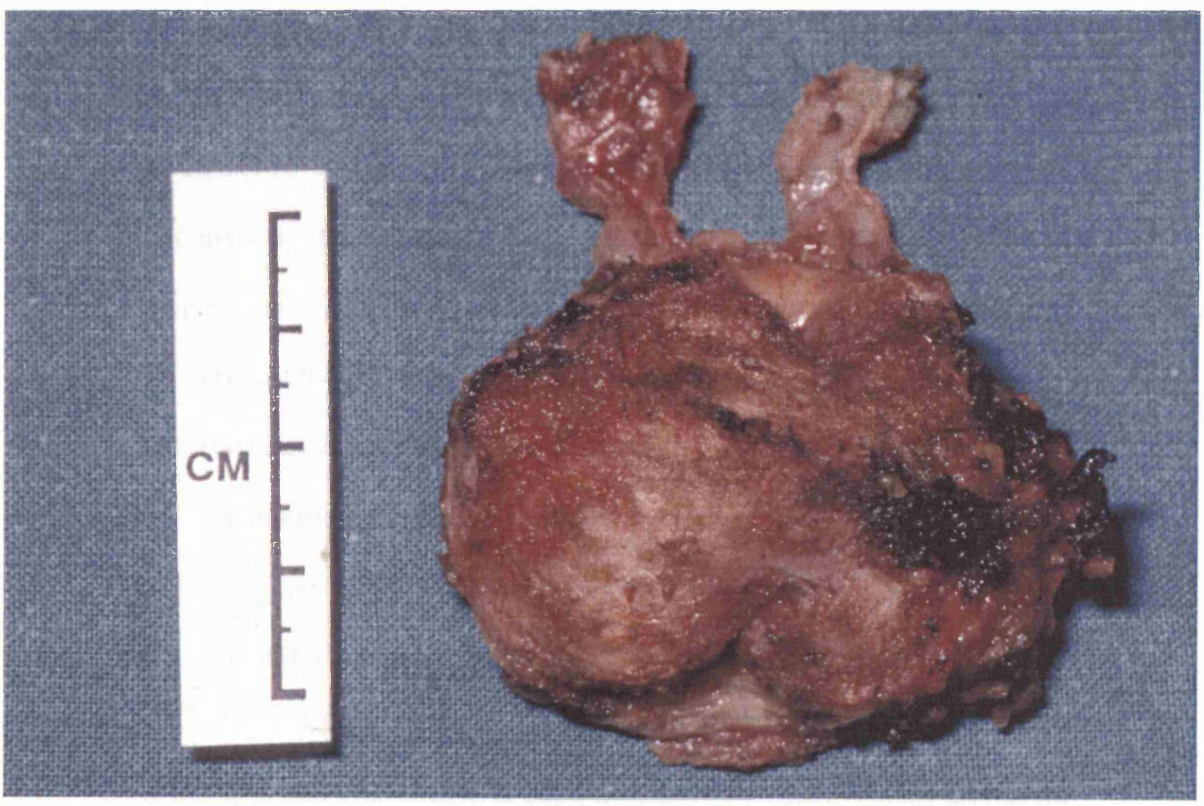

Fig.1: A prostatectomy specimen obtained from a retropubic radical prostatectomy.

A pseudo capsule that is indistinct from the surrounding fascial tissue surrounds the prostate. The surgical capsule is not a histological entity, but consists of an extension of prostatic parenchyma with dense fibrous tissue and smooth muscle that connect with the muscular layers of the prostatic urethra and cannot be separated from the prostate without tearing the glandular tissue. This tissue is thicker anterior and thins as it surrounds the prostate laterally and posterior. The pseudo-capsule has a number of areas of weakness:

i. The insertion of the neurovascular bundles
ii. The insertion of the ejaculatory ducts
iii. The insertion of the internal sphincter
iv. At the apex and in the region of the levator ani
v. And at the base where the bladder impinges upon the prostate.

At the apex, skeletal muscle is often mixed with prostatic glands and so the boundary is often obscure. This vagueness as to what constitutes the edge of the prostate has important implications for assessing extra prostatic extension by carcinoma or whether the capsule has been cut into.

The prostate is superior to the levator ani and is separated anteriorly from the pubic symphysis and the pubic bones by the veins of Santorini, fat, lymphatic, nerves and fascial tissues. Laterally the prostate is bound by the obturator internus. Posteriorly areolar tissue and Denonvillier's fascia separate the

prostate from the rectum. Cranially the prostate is attached to the bladder; the paired seminal vesicles are situated on the postero-superior aspect of the prostate separating the bladder from the rectum.

The caudal tip of the prostate, the apex, is where the urethra exits the prostate. The anterior aspect of the prostate is attached to the pubic symphysis and the pubis bones by the puboprostatic ligaments. The posterior surface of the prostate contains a central furrow the median sulcus. The prostatic urethra runs from the bladder to the prostate via the horse shoe shaped internal sphincter, which is at the base of the gland. It exits the horseshoe shaped external sphincter at the apex of the prostate where it becomes the membranous urethra. The prostatic urethra does not follow a straight line as it traverses the gland but bends anteriorly approximately 35 degrees at the verumontanum. There are two bilaterally paired ejaculatory ducts that pass from the junction of the medial vas and the lateral seminal vesicles, and anteriorly and inferiorly as they join the prostatic urethra at the verumontanum.

The internal sphincter is in the region of the proximal prostatic urethra and consists of urethral musculature with incorporation of both collagen and elastic fibres. The external sphincter at the level of the levator ani is composed of striated muscle. It surrounds the membranous urethra and extends into the prostate.

There are two significant groups of vessels in close relation to the prostate, the neurovascular bundles (described by Walsh) and Santorini's plexus. Santorini's plexus has minimal perforating vessels involving the prostate capsule. Its major feeding vessels are the deep vein of the penis, but there are some small tributaries from the prostate. The neurovascular bundle, as its name implies, is a mixture of the arteries, veins and nerves. These are bilateral, paired and run from the apex of the gland towards the tips of the seminal vesicles and the bladder. The bundles perforate the prostate capsule, and so allow a conduit for the spread of tumour at these potential sites of capsular weakness. The bundles were initially thought to be discrete structures at the base of the gland that could be dissected off. However recent work has suggested that this may be the case in the foetus, but in the adult as the prostate grows the ejaculatory nerves along with the prostatic fascia form a concave curtain around the prostate[234].

During surgery for the prostate the neurovascular bundles have a major implication with respect to the management of cancer and the potential morbidity associated with the operation. The other arterial structures are:

i) A basilar branch of feeding vessels course inferior to the seminal vesicles, enter the prostate at the base of the gland along the ejaculatory

ducts, and then courses to the level of the verumontanum.

ii) An apical branch, which enters the inferior portion of the prostate and courses to and feeds the surgical capsule.

iii) Urethral and inner gland flow is from a combination of the anterolateral and posterolateral vessels, coursing through the outer gland.

1.3 Lowsley's (lobar) anatomy:

The traditional description of the prostate divided it into five major lobes [231].

i) The anterior lobe, which is between the prostatic urethra and the anterior aspect of the prostate. This area arises from the ventral walls of the urethra.

ii) The median or middle lobe, which is defined as the area between the prostatic urethra anteriorly and the ejaculatory ducts posteriorly. This area arises from the rectal surface of the urethra and the lies above the orifices of the ejaculatory ducts.

iii) The posterior lobe which extends anteriorly from the ejaculatory ducts to the posterior surface of the gland. This area arises from the urethra below the ejaculatory ducts.

iv) and v) the two lateral lobes which are relatively symmetrical and arise from the lateral walls of the urethra.

The Lowsley classification is used everyday in clinical urology, however it is limited in its use as a means of interpreting histological anatomy.

1.4 McNeal's (Zonal) Classification:

This separates the prostate into the following four zones [248]:

i) The peripheral (outer – posterior and lateral)

ii) The central,

iii) The preprostatic region which encompasses the periurethral ducts and the transition zone

iv) The anterior fibro muscular stroma.

The anterior fibro muscular stroma consists of 33% of the prostate; it contains very few glands and consists of smooth muscle and dense fibrous tissue. The anterior fibro muscular stroma blends with the internal sphincter of the bladder neck proximally and the striated muscle of the external sphincter at the apex. The central zone surrounds the ejaculatory ducts with its apex at the veru montanum; this region is thought to originate from the mesonephric (Wolfian) duct, similar to the seminal vesicle. The peripheral and central zones are glandular (acinar), the majority of the prostate is composed of the peripheral region (75%). The peripheral zone is distal to the central zone and corresponds to a horseshoe shaped structure extending posteriorly, posteriolaterally and laterally. The majority of tumours arise in the peripheral zone, and the majority of benign disease occurs in the transition zone of the preprostatic region. The periurethral region consists of cylindrical smooth muscle

sphincter surrounding the urethra and extending from the verumontanum to the bladder neck. The transition zone consists of ducts, which extend laterally in the region of the verumontanum. The transition zone in health consists of only 5% of the normal prostate but it may grow considerably in benign glandular stromal hyperplasia.

This zonal classification is often simplified to a two-zone concept i.e.

i) inner (transition) zone, and

ii) outer (central and peripheral) zone.

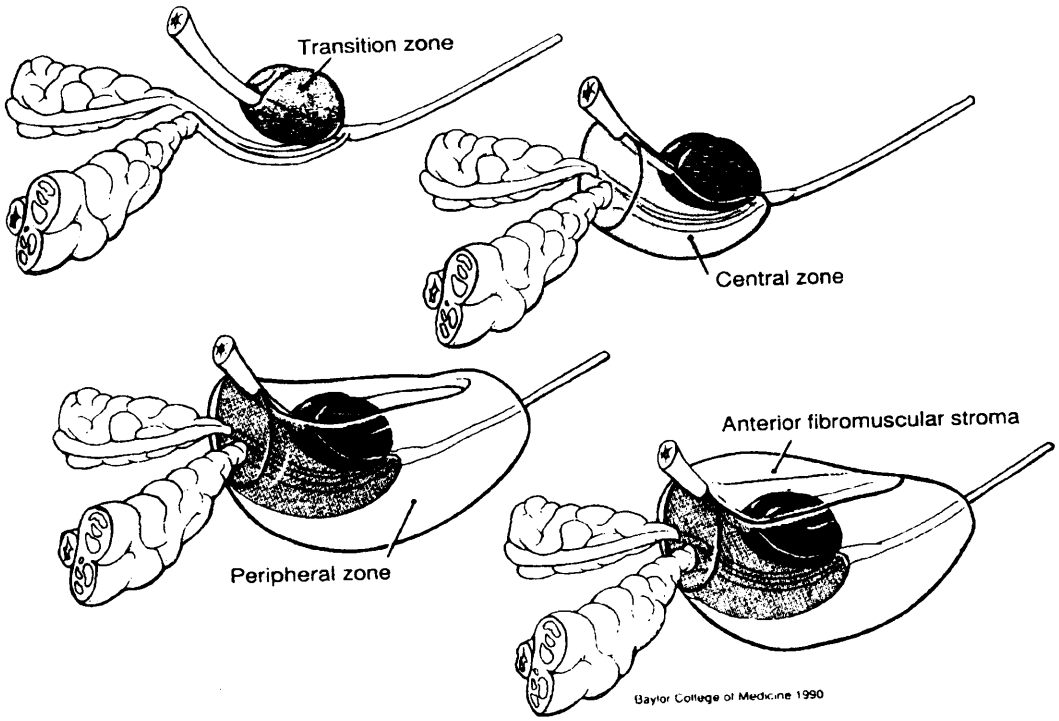

Figure2: McNeals Zonal Classification.

The transition zone surrounds the urethra proximal to the ejaculatory ducts. The central zone surrounds the ejaculatory ducts, and the peripheral zone consists of apical, posterior and lateral regions of the prostate[248].

1.5 Histology:

The prostate consists of stromal (smooth muscle cells, fibroblasts and endothelial cells) and epithelial cells. The epithelial cells line the alveoli. The epithelial cells consist of columnar secretory cells, basal cells and neuro endocrine cells. The tall columnar cells are secretory and are attached to each other by cadherins and to the basement membrane by integrins attached to laminin and fibronectin. These cells are terminally differentiated.

The basal cells lie beneath the secretory cells, and lie parallel to the basement membrane. The cells are elongated and are less differentiated than secretory cells. Basal cells are thought to be the progenitors of secretory cells. Basal cells are absent in adenocarcinoma of the prostate.

Neuroendocrine cells are present within the prostatic ducts and acini, and in the urothelium of the prostatic urethra [3]. These cells may function by endocrine, paracrine, neurocrine, and lumencrine mechanisms. They play an important regulatory role, both during growth and differentiation of the prostate and also in the secretory process of the mature gland.

1.6 Prostate Cancer

Fig 3: General Norman Schwarzkopf on the cover of Time, April 1996, having been diagnosed with prostate cancer.

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1.61 Epidemiology

In 2002, 8277 deaths occurred due to carcinoma of the prostate in England and Wales- the second commonest cancer related cause of death[282]. Data from the Office of Population Censuses and Surveys (OPCS) has recorded an increase in the incidence from 34.0 per 100,000 in 1980 to 79.3 per 100,000 in 2002. The OPCS believe that the increase in the incidence is not only due to better detection but also the underlying prevalence of the prostatic carcinoma is increasing. An ageing population coupled with an increased awareness amongst the medical profession and the lay public are also contributory factors. However an obvious disparity exists between the number of patients with carcinoma of the prostate and the number that succumb to the disease.

1.62 Aetiology

A number of risk factors have been implicated, age is the most important, 95% of cases occur between 45 and 89 years, the median age of presentation being 72; 90% of deaths occur after 65 at a median age of 77 years. Post-mortem studies have revealed 40% of 90-year-old patients have carcinoma of the prostate. Race is a risk factor, blacks are affected more than whites and Oriental's have the lowest prevalence. The highest prevalence is amongst black men in Washington DC[71].

An individual's risk is increased three fold if a first-degree relative has

carcinoma of the prostate. A group from the University of Utah is undertaking genealogy studies with the large Mormon population in Utah[252] and a group at the Royal Marsden is studying familial prostate cancer[111]. A high fat diet has also been implicated, as have androgens. Androgens are essential for gland proliferation and in experimental models induce carcinoma of the prostate. There is no clear relationship between serum testosterone levels and prostatic carcinoma but patients with low levels have a poor response to treatment.

1.63 Presentation

Carcinoma of the prostate presents principally in four ways. Patients present with symptoms of bladder outlet obstruction (this used to be the majority), secondly an incidental finding upon histopathological examination of prostatic chips, thirdly patients may present with metastatic disease, and finally screening whether it is specifically for carcinoma of the prostate or a routine examination. Among men with newly diagnosed carcinoma of the prostate one third have distant metastasis, 10-15% have locally extensive tumour (about half have lymph node metastasis), and 50-60% have clinically localised disease (a third have lymph node metastasis). Thus two-third of newly diagnosed patients have disease which is not localised and so is amenable only to palliation [340].

1.64 Diagnosis

Fig 4: The digital rectal examination still remains important.

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Three primary diagnostic tests exist to initiate histological or cytological tissue sampling: digital rectal examination (DRE), trans-rectal ultrasound (TRUS), and prostate specific antigen (PSA). The clinical impression of the examining urologist correlates well with the positive predictive value of DRE. On biopsy samples from clinically normal prostates a positive predictive value of 2% was calculated, compared to 83% on those with marked induration [363].

Transrectal ultrasound is the commonest modality used to image the prostate. It is of limited benefit with a distinctly palpable lesion. However when the DRE is unremarkable and the PSA is raised the positive predictive value of a TRUS guided biopsy is 16.4%. If the PSA is normal and the DRE is abnormal it is 13%, and if both tests are unremarkable it falls to 9.3%[88]. If DRE is abnormal and a digitally guided biopsy result is benign then in the same cohort a TRUS guided biopsy will be positive in 53% of patients [164]. The addition of sextant biopsies further improves the detection rate [165] and more recent work has shown that increasing the number of biopsies as a factor of the gland volume leads to further improvements in detection rate[104:237]. Up to six sets of biopsies may be necessary to rule out cancer of the prostate[324].

Prostate specific antigen is the best marker for prostatic carcinoma and probably the best tumour marker currently available. It is an established

immunohistochemical marker and is used to monitor patients. It can also be used to aid staging and diagnosis, and proponents of screening strongly advocate its use as a screening tool. PSA is a serine protease secreted into the ductal system of the prostate where it is thought to catalyse the liquefaction of the seminal coagulum after ejaculation. However, it does not explain the clotting and liquefaction of semen, which is so important for reproduction. Any process that disrupts the normal architecture of the prostate allows diffusion of PSA into the stroma; here it gains access to the blood stream via the stromal micro vasculature. Elevated levels of serum PSA are detected in prostatitis, infarcts, benign prostatic hypertrophy, transiently after prostatic manipulation and with prostatic adenocarcinoma. Cancer produces less PSA per cell than benign epithelium but the greater number of malignant cells and the stromal disruption associated with cancer account for the clinically important elevation in serum PSA levels [34]. The higher the grade of the cancer, the less differentiated the cells and the less PSA is produced with Gleason grade 5 tumours contributing relatively little PSA to the serum PSA[5]

A number of PSA parameters have been described to improve its sensitivity, specificity, and clinical utility. The normal range of PSA is less than 4.0ng/ml, however in 20% of patients with newly diagnosed prostatic carcinoma the PSA levels are less than 4.0 ng/ml. PSA may increase with age as a result of benign hypertrophy. Therefore, age specific reference ranges (Table 1) have been

introduced and appear to increase the sensitivity of PSA in men younger than 60 years and the specificity in men older than 60 [290].

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Age yr.	40	45	50	55	60	65	70	75	80
PSA ng/ml	2.0	2.4	2.8	3.3	3.8	4.5	5.3	6.2	7.2

TABLE 1: Age-specific reference ranges for PSA.

PSA density, i.e. serum PSA divided by the volume of the prostate, was introduced to adjust for contribution of benign prostatic hypertrophy to serum PSA levels, it was found to be a better predictor of pathological stage than the Gleason grade [26;347]. Others have not found this parameter useful[51;290].

A PSA velocity, i.e. the rate of increase of PSA over time, greater than 0.75 ng/ml per year is an accurate predictor of cancer identifying 72% of cancer cases with a specificity of 90% [69]. In cohort studies serum PSA correlates with cancer volume, tumour grade and pathological stage[300 34], but it is not reliable on an individual basis.

If PSA is used as the initial test in asymptomatic men more than 50 years of age, then with the aid of TRUS guided biopsies 22% of men with a PSA of 4.0 - 9.9 ng/ml are diagnosed as having carcinoma of the prostate. This figure increases to 67% in men with PSA > 10.0ng/ml [72].

Any of the three primary investigations can lead to a tissue biopsy, but PSA is the most sensitive single parameter [72] and the positive predictive value of DRE and TRUS can be increased with a raised PSA.

1.65 Staging of Prostate Cancer.

Once the diagnosis of prostatic carcinoma is made, staging is carried out with

radiological imaging, a nuclear bone scan and TRUS. As part of the WHO consensus conference on imaging it was concluded that MRI is superior to other modalities in the assessment of local tumour stage, particularly if an endorectal coil was used [167:242]. The majorities of lesions arise in the peripheral zone and are seen as low signals on T_2-weighted images. The accuracy of MRI in differentiating invasive from localised disease is well established. A peripheral zone defect of 1cm or greater with an ill defined border and of low signal proved to be 100% sensitive [306] and 54% specific [309] for extra capsular spread (fig. 5).

Fig 5a **Fig5b**

Fig 5: Trans abdominal MRI of the prostate, a large left sided cancer can be seen in the peripheral zone. Fig 5a shows a T2 cancer and Fig 5b a T3 lesion. Although the images obtained with transrectal MRI were initially better, improvement in MRI technology has allowed extra corporal MRI to supersede this.

The value of MRI in detecting nodal disease was limited to evaluation of lymph node size and its accuracy, i.e. 80-90%, has not exceeded that of CT [31]. Any node greater than 1.5cm is likely to be malignant. However more recent work with ultra small particles of iron oxide – ferumoxtran-10, in pelvic malignancies has shown that the sensitivity may be increased with no loss of specificity [323]. It remains to be seen if this is of any benefit in the detection of pelvic lymph nodes in patients with prostate cancer.

Ultrasound imaging of the prostate via the abdominal approach is unreliable, the use of the transrectal probe has allowed excellent high resolution images of the peripheral zone where 70% of the carcinomas arise [243], however it should be remembered that 24% of prostatic tumours are isoechoic [95]. Transrectal ultrasound detection of local extension is operator dependent and the accuracy varies from 60 to 100% [322;339].

The utility of CT is diminishing. It may have a use in situations where MRI scans are inconclusive, e.g. the assessment of para-aortic nodes.

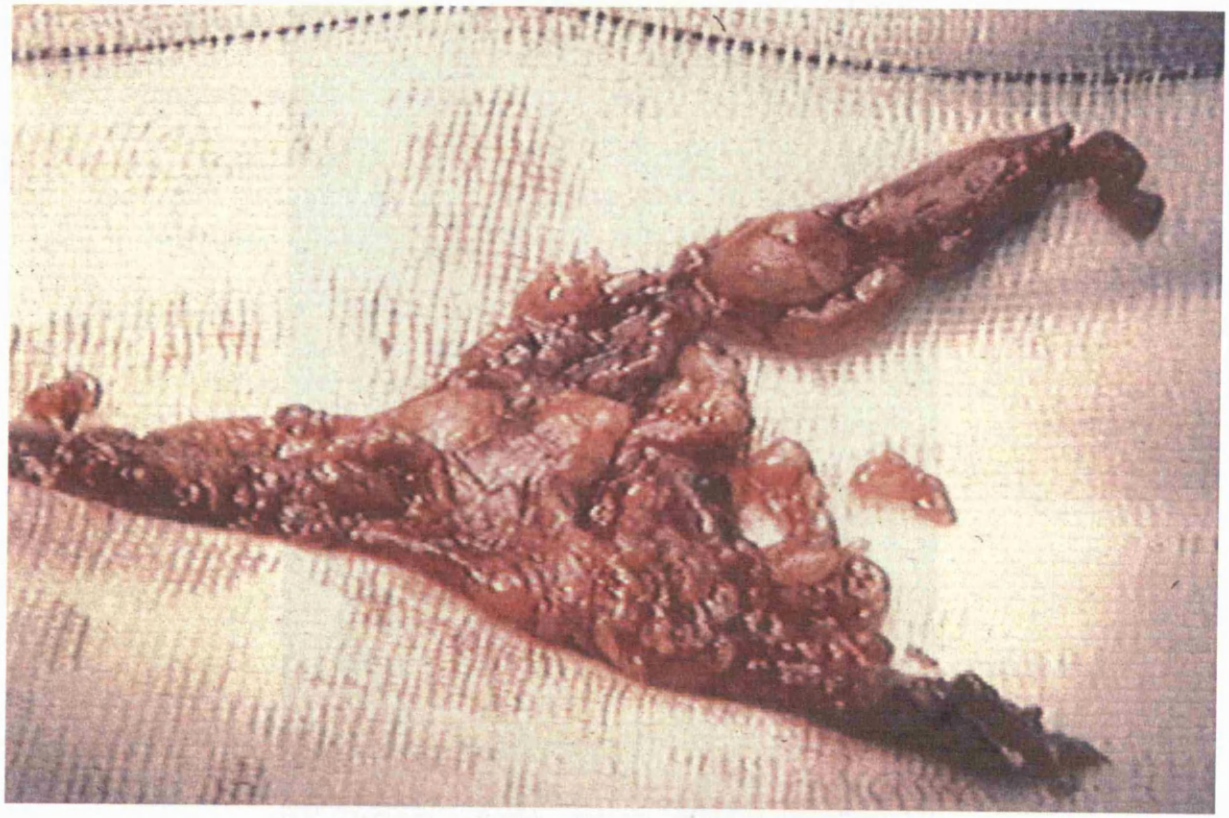

Fig 6: Lymph nodes obtained by surgical dissection.

Fig 7: A positive bone scan in a patient with multiple metastasis.

The axial skeleton is the commonest site of haematogenous metastasis. Bone metastasises are assessed with scintigraphy (fig.7) that can point to the need for plain radiographs. Routine radiographic surveys have no place in imaging for skeletal involvement; it is costly, time consuming and involves a significant dose of radiation. Furthermore there must be a 50% loss of bone density before a lesion is visible[221] and 23% of patients judged to be free of bone metastasises by radiography have metastatic disease by scintigraphy [303].

The mainstay of assessing chest involvement is a chest radiograph [242].

The level of serum prostate specific antigen is determined by prostate cancer volume and by the percentage of high-grade cancer [188]. Therefore, the use of serum PSA coupled with tumour grade and clinical stage has been advocated by Partin et al as a staging tool [302]. In a series of pelvic lymphadenectomies if the PSA < 6ng/ml and the Gleason score < 5 all the pelvic lymph nodes were tumour free (n=142), but if the PSA < 10 ng/ml and the Gleason score < 6 then 1% of the pelvic lymph adenectomies were positive (n=388) [281].

Once the localised tumour has been staged using the TMN classification, (Table2) the patient is a candidate for one of the three management regimes available:

i) watchful waiting

ii) active surveillance,

iii) radiotherapy - external beam

brachytherapy

iii) radical prostatectomy- retro pubic,

perineal, and

laparoscopic.

TABLE 2: Staging of Localised Prostate Cancer (UICC 2002).

T	Primary Tumour
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
T1	Clinically inapparent tumour not palpable or visible by imaging.
T1a	Clinically inapparent tumour not palpable.
T1b	Tumour incidental histological finding in more than 5% of the tissue.
T1c	Tumour identified by needle biopsy.
T2	Tumour confined within the prostate or invading the apex.
T2a	Tumour involves one half of one lobe or less.
T2b	Tumour involves more than half of one lobe, but not both lobes.
T2c	Tumour involves both lobes.
T3	Tumour extends through the prostatic capsule.
T3a	Unilateral or bilateral extra capsular extension.
T3b	Tumour invades the seminal vesicles.
N0	No regional lymph node metastasis.
M0	No distant metastasis.
Nx	Regional lymph nodes not sampled.
N0	No positive regional lymph nodes.
N1	Regional lymph node metastasis
MX	Distant metastasis cannot be assessed.
M0	No distant metastasis.
M1	Distant metastasis.

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1.66 Management:

Watchful Waiting.

Patients are initially followed without treatment and receive subsequent treatment for symptoms e.g. androgen ablation for metastatic disease or transurethral resection for outlet obstruction. Limited data is available from 4 studies about the outcome of watchful waiting (Table 3).

TABLE 3:

Localised prostate cancer, deferred therapy: Progression, Metastatic Rate, and Prostate Cancer Death.

Author	No. of patients.	Mean age	5 years/% Prog.	Met.	Death
George[126]	120	75	84	11	20
Johansson[184]	223	72	31	9	8
Whitmore[415]	75	67	69	37	0
Adolfsson[4]	122	68	55	14	1

These studies demonstrate that within 5 years 31-84% of patients will progress and that metastasis will occur in 9 to 37% of patients. A pooled analysis of six non randomised studies, which included three of the four studies listed in table 3 [4;184;415], concluded at 10 years the disease specific survival for low grade disease (grade 1+2) was 87% with a metastasis free survival rate of 81% for grade 1, and 58% for grade2. The figures for high-grade (grade 3) disease were 34 % and 26% respectively[82].

A more recent study from Denmark of 2,570 patients with newly diagnosed clinically localised prostate cancer treated with watchful waiting; radical prostatectomy was not performed in Denmark before 1995, and external beam radiotherapy was only rarely used; demonstrated an annual mortality of 13%, with an increased in mortality for all ages at follow up. Follow up was at 10-14 yrs, 15-19 yrs, and greater than 20 years after diagnosis[49]. Additionally the results of the Medical Research Council immediate versus deferred treatment study for carcinoma of the prostate have demonstrated more rapid progression and death in the deferred treatment group for all stages [94].

1.67 Management: Active surveillance.

The rational behind this management option is that the majority of patients have a disease which is indolent and will not progress, and to treat these patients with curative intent would lead to unnecessary morbidity without any

survival benefit. Therefore active surveillance, to paraphrase Whitmore, attempts to separate the pussycats from the tigers. Patients with localised organ confined disease are followed up with three monthly reviews including PSAs DREs, and repeat biopsies, as part of a study protocol, no intervention is required unless there is evidence of disease progression – progression in PSA, Gleason score, or clinically.

If there is evidence of progression or it is felt that the patient is not suitable (high Gleason score) for active surveillance, the patient is referred for curative therapy. This is distinct from watchful waiting, which relies on the use of palliative intervention for symptomatic progression[157:297].

1.68 Management: Radiotherapy - External Beam:

The Stanford series consists of 1119 patients (from October 1956 to December 1990) treated with external beam irradiation derived from a mid to high-energy linear accelerator (range, 6-25 mV). 673 patients had disease confined to the prostate [14]. These authors found survival to be inversely proportional to clinical stage and pathological grade. At 15 years the survival rates for the best prognosis tumours were equivalent to that of an age matched cohort i.e. 50% at 15 years, however the poorer prognosis tumours had a survival rate of 18% at 15 years. Age had no influence on outcome.

More recently the use of adjuvant androgen blockade along with external beam radiotherapy has been championed. In a randomised study, androgen ablation was started at the time of the first dose of irradiation and continued for three years after. In patients with locally advanced disease, there was a survival advantage and decreased local progression in the group on androgen ablation therapy[37;38].

1.69 Management: Radiotherapy - Brachytherapy:

In this form of radiotherapy, radioisotopes are inserted directly into the prostate. It has been used for the management of other malignancies arising in organs accessible to this type of delivery system including the brain and the gynaecological tract. The Seattle group has data from 634 consecutive patients who have undergone brachytherapy[36], with 10 year follow up available on 125 patients[140]. The initial results appear to be similar to those obtained for external beam radiotherapy or surgery. Brachytherapy is not a benign form of management. 5-10% of patients develop short term urinary retention[36], the Seattle group report incontinence rates of less than 1% and potency rates of 75 to 90% after treatment[36]. Other series have not been so flattering reporting 40% of patients developing urinary incontinence, with 18% reporting having used a pad in the last week [378]. The risk of incontinence appears to increase with time and 83% of men who had had a previous TURP were incontinent[378]. The incidence of total impotence is 45%, with 68% of men having an erection

inadequate for penetration [378].

1.70 Management: Radical Total Prostatectomy.

Surgery is curative only if the entire tumour is removed. Two approaches are advocated for open surgery, retro-pubic or perineal, the results are comparable. Precise control of bleeding from the dorsal vein complex allows anatomic dissection of the apex, and thus identifies the branches of the pelvic plexus which innervate the corpora and so preserves sexual function [409]. Incontinence (6-10%) and impotence (30%) are serious side effects.

Laparoscopic radical prostatectomy is slowly gaining favour in the United Kingdom. Richard Gaston developed the initial technique in Bordeaux; however this remains an unpublished series. The procedure was popularised by Guillonneau and Vallencien[143-145]. The Montsouris group advocated a transperitoneal approach. This technique is based on the primary incision of the peritoneum above the recto-vesicle cul-de-sac followed by dissection of the seminal vesicles. This approach does not adhere to the basic laparoroscopic tenet of replicating the open operation. Therefore, an alternative extra peritoneal approach has been developed[39].

Whichever approach is adopted, open or laparoscopic, perineal or retro pubic, transperitoneal or extra peritoneal, oncological cure coupled with acceptable

functional results remains the imperative. To this end, data from the Virginia Mason Clinic for patients with localised disease revealed a 15 year crude survival of 64% and after 10 years the disease specific mortality was 2% with the cause specific survival curve plateau at 17 years with no further disease specific mortality [128]. The group from Duke, Durham demonstrated a significant difference in the failure and death rates between patients with organ confined, specimen confined, and margin positive disease, i.e. failure rates of 12%, 30%, 60%, and death rates of 8%, 12%, and 30% at 10 years respectively [304]. A more recent series has shown that the grade of the tumour also affects the rate of progression[113]. Therefore cancer control after surgery is affected by tumour grade, and whether the tumour was organ-confined, specimen-confined, or not confined to the specimen [113]. PSA can also be a measure of recurrence however biochemical PSA failure may not manifest itself as clinical progression in the patient [113].

Other novel approaches have also been advocated, such as high intensity focussed ultrasound [35] and cryotherapy[28], but long term data is awaited.

Advocates of screening in Britain suggest there may be a potential benefit in terms of survival if screening is adopted [298], however there is considerable opposition to this in this country. In the USA early detection programmes detect organ-confined disease in a younger cohort of patients [341]. Long-term

follow up of these patients is required to determine if there is an improvement in survival. A study comparing two large fixed cohorts of male Medicare patients, Seattle n = 94,900 and Connecticut n = 120,621, exposed to different screening regimens concluded that more intensive screening over a eleven year period is not associated with lower prostate cancer specific mortality [232]. A recent randomised study from Denmark compared surgery with watchful waiting and initially demonstrated a significant improvement in cancer specific survival in the surgery arm but no improvement in overall survival [169], a further update has now shown a decrease in local progression and a decrease in the development of distant metastasis in the patients randomised to surgery[33].

Using the SEER (surveillance, epidemiology, and end results) data base a study of 104,577 patients with localised prostate cancer concluded that 1) treatment is associated with lower disease specific and overall mortality, and 2) advanced age and black race are markers of disease specific and overall mortality. No conclusion was drawn about the optimal treatment modality [206].

A second study using the SEER database and an intention to treat analysis i.e. patients with lymph node dissection and radiotherapy or radiotherapy alone were assigned to the radiotherapy group, and those with a lymph node dissection alone or radical prostatectomy were classified into the prostatectomy group. The advantage of this type of analysis is that it includes

patients in the prostatectomy group who have been deselected by pathological staging. The disadvantages are that co-morbidity and tumour-related variables, e.g. tumour size and PSA, are not controlled for. The data suggested that treatment was most useful for high grade tumours, patients with low grade tumour did better than the general population, and the data for surgery may be flattering because surgeons were the selecting best patients when analysing data by treatment received [233].

The Prostate Cancer Clinical Guidelines Panel has recently published its report on the management of clinically localised prostate cancer [257]. The report concluded that the published data to date was inadequate for a valid comparison of the treatment options and, all patients with newly diagnosed, clinically localised cancer should be informed of all the treatment options. The appropriate patient for total prostatectomy should have organ confined disease, an expected longevity that is longer than the natural history of the cancer i.e. 15 years, no significant surgical risk factors and a preference to undergo surgery. The major advantage is the potential for removal of the cancer. The downside includes genitourinary dysfunction and disease progression. The patients most likely to benefit from radiation therapy would have a relatively long life expectancy. No significant risk factors for radiation toxicity and a preference for radiation therapy. In favour of radiotherapy is the fact it is well tolerated and a potential for cure exists. The adverse effects include radiation

cystitis, proctitis, and erectile dysfunction. The prostate is still in place and so disease progression may occur. Patients with a short life expectancy and/or a low-grade tumour are likely to be suitable for surveillance. Disease progression is likely with only a marginal compromise to disease specific survival at 5 and 10 years [257].

The absence of hard data is a constant source of embarrassment to all health professionals involved in the management of localised carcinoma of the prostate. The Department of Veterans Affairs and the National Cancer Institute's Cancer Therapy Evaluation Programme Co-operative Study (407)- the Prostate Cancer Intervention Versus Observation Trial (PIVOT) - is a randomised trial designed to determine the benefits of early intervention with radical prostatectomy compared to surveillance[418]. PIVOT will enrol 2000 patients over a three year period and follow up will be for a minimum of 12 years. Radiotherapy has not been included as a third treatment arm because of sample size, cost, and feasibility. This study should hopefully answer some of the questions.

In the United Kingdom, the three arm randomised ProtecT (prostate testing for cancer and treatment) randomised study is currently recruiting [107]. This study has demonstrated the negative connotation patient's place on the phrase watchful waiting, patients interpreted this as no treatment, and has replaced

this with the acceptable concept of active monitoring.

The peculiar biology of this disease has contributed to the lack of a consensus about the management of localised prostate cancer. The median time of detection of this disease is 72 years, the median age of death due to prostate cancer is 77 years, since the average life expectancy is 74.2 years, and it is evident that the most men will die in spite of the disease rather than because of it.

Chapter 2: Prostatic Intraepithelial Neoplasia.

The increasing focus of the public has generated considerable interest in the management of prostate cancer. This in turn has resulted in screening programmes being established; not only have these programmes detected prostate cancer but they have detected an increasing number of men with atypical epithelial proliferation the most common being prostatic intra-epithelial neoplasia.

2.1 Prostatic intra-epithelial neoplasia

Bostwick and Brawer [46] introduced the term Prostatic Intraepithelial Neoplasia (PIN) in 1987, and although initially it was divided into three grades it rapidly evolved into a two tier system – high and low grade [137]. Low grade PIN is of no clinical relevance and the generic term PIN exclusively refers to high grade PIN. In a model analogous to Vogelstein's for colorectal cancer, it has been proposed that PIN refer to the precancerous end of the morphological spectrum. PIN is defined as an abnormal proliferation, where the cellular changes within the prostatic ducts, ductules and acini exhibit the cytological changes of cancer i.e. progressive loss of the markers of secretory differentiation, increasing nuclear and nucleolar abnormalities, increasing proliferative index, neovascularisation, genetic instability, and variation in

DNA, but retain a basal cell layer (intact or fragmented). PIN is a spectrum of atypical cytological features ranging from minimal change to those that are indistinguishable from prostate cancer. The most striking features are nuclear and nucleolar enlargement, and are often regarded as diagnostic of PIN. The absence of a basal cell layer distinguishes cancer from PIN. The generic term PIN usually refers exclusively to high grade PIN. Low grade PIN is no longer thought to be clinically relevant.

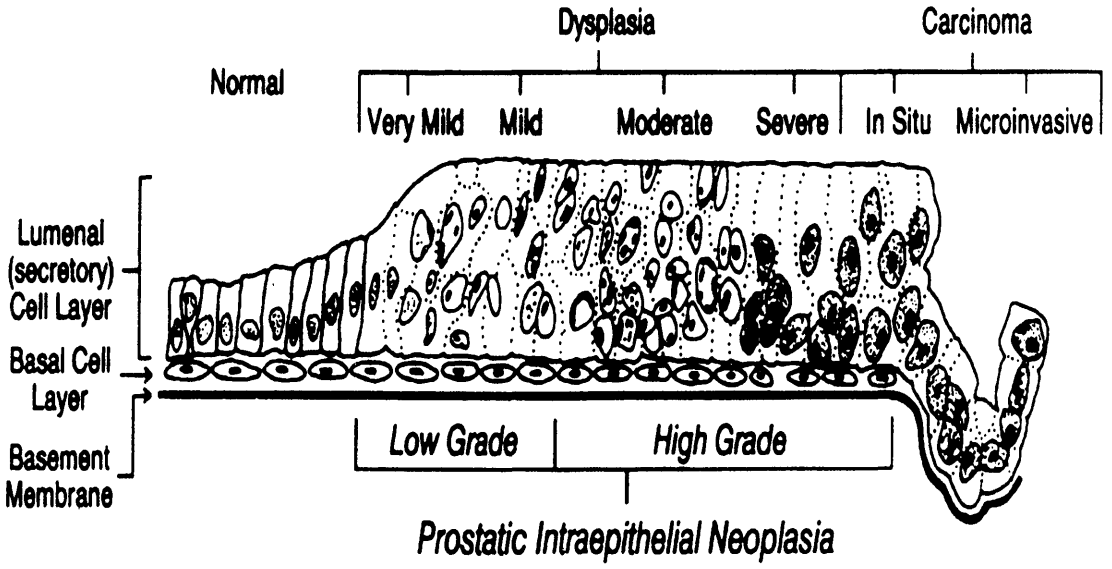

Figure 8: The continuum of change from benign to malignant proposed by Bostwick and Brawer [46]. The loss of the basal cell layer is pathognomonic of cancer, but the the cytological changes of cancer occur in prostatic intraepithelial neoplasia which has an intact basal cell layer. The presence of the basal cell layer can be confirmed with the use of antibodies against high molecular weight cytokeratins.

There are four main architectural types of high grade PIN: tufting, micro papillary, cribriform, and flat [45].

Fig 9: Tufting PIN (low power).

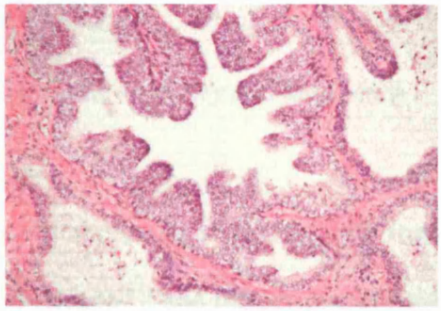

Fig10: Micropapillary(low power).

Fig 11: Cribriform PIN (low power)

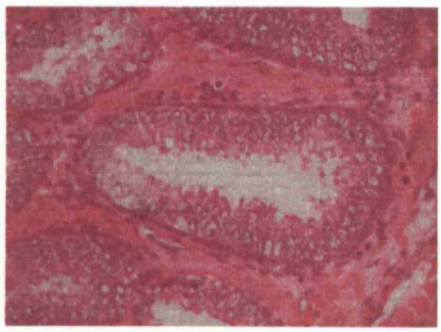

Fig 12: Flat PIN (low power).

These different architectural types do not appear to have any clinic pathological significance. PIN spreads through the prostatic ducts in three different patterns [44]:

i) Neoplastic cells replace the normal luminal secretory cells,

ii) Direct invasion of the duct wall,

iii) Pagetoid spread, the cells grow between the secretory and basal cells.

Clinical studies suggest that PIN precedes carcinoma by ten years, with low-grade PIN first emerging in men in the third decade [331] and the peripheral zone is the most common location for PIN. The frequency of PIN in prostates with adenocarcinoma is much greater than in prostates without cancer [311].

	Low-grade PIN (PIN 1)	**High grade PIN (PIN 2 and 3)**	**Cancer**
Architecture	Epithelial cell crowding and stratification, with irregular spacing.	More crowding and stratification. Four patterns: tufting, micro papillary, cribriform, and flat	Crowding with stratification
Cytology: Nuclei	Enlarged with size variation	Enlarged with size and shape variation.	Hyper chromatic nuclei with macro nucleoli.
Cytology: Chromatin	Normal	Increased density and clumping	Increased density and clumping
Cytology: Nucleoli	Rarely prominent	Frequently large and prominent	Large and prominent
Basal Cell Layer	Intact	May show some disruption	Absent
Basement Membrane	Intact	Intact	Absent

Table 4: Summary of relationship between prostatic intraepithelial neoplasia and cancer.

Prostatic Intraepithelial Neoplasia and Prostate Cancer.

2.2 Incidence of high-grade PIN in benign prostate tissue (biopsy and TURP)

The incidence of PIN varies in benign needle biopsy specimens between 0.7% and 16.5%. In TURP specimens it is between 2.8% and 3.2%. The incidence of PIN is lower in screened populations than in patients presenting for urological assessment.

Ref./Year	No of Patients	Tissue Specimen	Patient	Incidence of PIN /%
[218]/1989	256	14 G needle biopsy	Non-screening	11
[253]/1991	330	18 G needle biopsy	Screening	5.2
[321]/1994	163	18 G needle biopsy	Non-screening	8.6
[48]/1995	200	18 G needle biopsy	Non-screening	16.5
[48]/1995	200	18 G needle biopsy	Non-screening	9.5
[216]/1996	1275	18 G needle biopsy	Non-screening	4.4
[417]/1997	439	18 G needle biopsy	Non-screening	5.5
[360]/1997	79	14 G needle biopsy	Non-screening	7.6
[305]/1997	148	18 G needle biopsy	Non-screening	14.1
[80]/1997	1009	18 G needle biopsy	Non-screening	1.5
[124]/1997	158	TURP	Non-screening	3.2
[296]/1997	570	TURP	Non-screening	2.8
[318]/1998	2219	18 G needle biopsy	Non-screening	4.0
[172]/1998	388	18 G needle biopsy	Non-screening	8.0
[320]/1998	795	18 G needle biopsy	Non-screening	7.4
[294]/1998	62537	18 G needle biopsy	Non-screening	4.1
[287]/1999	15753	18 G needle biopsy	Non-screening	3.9
[166]/1999	8763	18 G needle biopsy	Screening	.7
[332]/2000	3891	18 G needle biopsy	Non-screening	5.4
[8]/ 2000	485	18 G needle biopsy	Non-screening	6.8
[170]/2001	1474	18 G needle biopsy	Screening	4.7
[120]/2001	1961	18 G needle biopsy	Screening	4.7
[284]/2004	1086	18G needle biopsy	Non-screenining	4.0
[267]/2005	1188	18G needle biopsy	Non-screenining	2.7

Table 5: The incidence of PIN according to recent studies.

The incidence and extent of PIN appears to increase with age, it is more prevalent and extensive in patients of African origin[120]. PIN is also detected a decade earlier in men of African origin compared to Caucasians[120].

2.3 Location of PIN

PIN lesions are often multifocal, and bilateral, as is prostate cancer. PIN and prostate cancer are principally located in the peripheral zone of the gland. In radical prostatectomy specimens with cancer, high grade PIN is present in the transition and peripheral zone simultaneously in 36% of cases, and is found exclusively in the transition zone in 1% of cases[311].

2.4 PIN and serum PSA levels

This is a contentious area; a study of 81 men undergoing surgery for benign disease found that in men with PIN alone the mean serum PSA fell between the levels for men with benign and malignant disease[50]. The same authors found similar results for patients with BPH, PIN, and cancer on TRUS sextant biopsy [52]. However, Ronet et al [326] and Alexander et al[6] did not confirm this finding. In a recent study of 81 consecutive men who have undergone radical retro pubic prostatectomy it was shown that PIN did not contribute to total or percent free PSA levels[269]. Using grid counting to calculate the volume of tumour, PIN, and benign disease, in a study of 194 patients PIN volume was

shown to correlate to PSA, this result was interpreted by the authours as an artefact and they concluded that PIN did not to contribute to it[6]. Immunohistochemical studies reveal that the expression of PSA in PIN is less than that observed in BPH and cancer[249]. The explanation for a lower PSA in patients with PIN is that PIN is an abnormality within pre-existing ducts and acini, and cytoplasmic secretory products would be expected to wash downstream rather than escape into the stroma and blood vessels.

2.5 PIN and Radiotherapy.

A study of 29 patients who underwent salvage prostatectomy after radiotherapy demonstrated PIN in only two of the specimens. After radiotherapy the basal cell layer in PIN becomes more prominent and the presence of cytoplasmic blebs distinguishes secretory cells from basal cell. [77].

2.6 Clinical Significance of PIN

High grade prostatic intraepithelial neoplasia has a high predictive value as a marker for adenocarcinoma, Davidson et al evaluated this in a retrospective case control study of 100 patients with needle biopsies with high grade PIN and 112 biopsies without PIN matched for clinical stage, patient age, and serum PSA. Adenocarcinoma was identified in 35% of the subsequent biopsies with PIN compared with 13% in the controls. The likelihood of adenocarcinoma increased with time, 32% at one year, and 38% after one year.

The presence of high grade PIN and a raised PSA are associated with adenocarcinoma in 51% of cases in repeat biopsies [192]. Others studies have found cancer detection rates of 28% [216] and 47% [350].

The incidence of prostate cancer after an initial diagnosis of PIN increases if a patient has repeat biopsies[207]. In a series of 245 patients with an initial diagnosis of PIN, cancer was diagnosed in 24.5% of patients on the initial repeat biopsy and a total of 32.2% after the second repeat biopsy [207].

Recent work has also highlighted the importance of second biopsies with a diagnosis of PIN. In a cohort of 190 men diagnosed with PIN and having serial repeat biopsies the overall incidence of prostate cancer on repeat biopsies was 30.5%, but if there was a second biopsy with PIN detected, the risk of cancer increased to 41%, compared to only 18% if the second biopsy had benign tissue[135].

PIN is often associated with moderately to poorly differentiated adenocarcinoma of the prostate; it is not often associated with low-grade prostate cancer or cancer arising in the transition zone [263].

2.7 Evidence linking PIN and Cancer.

There is no disruption of the basal cell layer in benign disease, where as in

cancer the basal layer is absent. In PIN there is partial disruption. There is increased expression of the proteolytic enzyme type IV collagenase in cancer and PIN compared to benign disease[264].

The number of stromal micro vessels in PIN is greater than in benign or BPH tissue, but less than in adenocarcinoma. The micro vessels in PIN are shorter than those in benign epithelium, with irregular contours and open lumina, an increase in the number of endothelial cells, and a greater distance from the basement membrane[264].

The doubling time of PIN is at an intermediate level when compared to BPH and cancer [265] and there is a progressive increase in the number of mitotic figures and apoptotic bodies from BPH through to PIN and finally cancer[266].

The cell kinetics of PIN have been studied. It has been shown that PIN, which is not associated with carcinoma, shows a down regulation in apoptosis, which is common with prostate cancer, but distinct from PIN which is associated with prostate cancer [200]

Many markers of differentiation are down regulated in PIN and cancer, e.g. neuroendocrine cells[47], prostatic acid phosphatase, and prostate specific[96] antigen. Benign prostate has most cells with neuroendocrine differentiation.

Others are up regulated e.g. fatty acid synthetase[277], glycoprotein A-80[136], glycoprotein TAG-72[54], and Lewis X and Lewis Y antigen[276].

Using micro array analysis hepsin is up regulated in PIN, and prostate cancer but is absent in benign tissue[101], in hormone refractory disease it has an intermediate level of staining. Hepsin has been shown to be a predictor of relapse[101]. PIM1 kinase was up regulated in all stages of prostate cancer but absent in PIN and benign disease[101].

Transgenic mice have been used to study the pathogenesis of prostate cancer. The most extensively used model is the TRAMP (transgenic adenocarcinoma mouse prostate) model[130 138]. The simian virus 40 (SV40) early region tumour antigens have the ability to induce transformation in vivo. The large T antigen (Tag) interacts with the retinoblastoma gene (Rb) and p_{53}, and the small t antigen interacts with a protein phosphatase. The loss of wild type Rb and p_{53} was used in this model. Probasin was used to target the SV40 to the prostate. Probasin encodes for an androgen and zinc related protein specific to the dorsolateral epithelium of the prostate. A probasin SV40 Tag construct was used to target the prostate of mice [138]. The expression of SV40 early genes in the prostate of the mice results in mice displaying progressive forms of prostate disease. The mice develop prostatic hyperplasia at 10 weeks and invasive adenocarcinoma by 18 weeks of age. By 10 to 12 weeks TRAMP

mice reproducibly displayed evidence of high grade PIN [129]. By 30-36 weeks all TRAMP mice developed metastases[130]. This model has therefore facilitated in the understanding and characterisation of the sequence of events, which may occur in the initiation, and progression of prostate cancer. This model is however open to criticism, since in the human form of the disease benign prostatic hyperplasia does not progress to adenocarcinoma.

2.8 Differential diagnosis of PIN

There are several benign conditions that can mimic PIN; these include atypia induced by inflammation, infarction, or radiation; transitional cell metaplasia; basal cell hyperplasia; clear cell cribriform hyperplasia; normal ejaculatory duct and seminal vesicle epithelium. Malignant lesions, which have to be distinguished from PIN, include high grade transitional cell carcinoma involving the prostate ducts and acini, and cribriform acinar, and cribriform ductal prostatic carcinoma[299].

Despite its major impact as the most common cancer in men, prostate cancer is relatively poorly understood at the genetic level. It is hypothesised that, as with other neoplasms, mutations of proto-oncogene and tumour suppressor genes contribute in some way to the genesis of prostate cancer. The use of molecular markers to supplement clinical information concerning the biological aggressiveness of a carcinoma may allow better selection for

different management strategies. Alternatively, they may be used after surgery to stratify men into groups whereby those with poorer prognosis may receive adjuvant treatment, perhaps providing an advantage of improved disease specific survival.

Chapter 3: P_{53}, Bcl_2, Bax-$_{alpha}$, and Ki-67.

3.1 P_{53}

The p_{53} gene is located on the short arm of chromosome 17 (17p13.1). The gene is composed of 11 exons. The p_{53} gene product is a 53Kda (393-amino acid) nuclear phosphoprotein [203], which inhibits the advancement of cells from the G1 to the S phase. Lane and Crawford first identified this in 1979 [214].

The DNA tumour virus SV40 expresses two antigens, small t and largeT. These are essential for the virus to transform cells. Lane and Crawford demonstrated that the T antigen binds to a 53 Kd host protein, and precipitated this out. They postulated that this protein was not virally coded and that it could be detected in cells transformed by other viruses, radiation, chemical carcinogens, or even in untransformed cells. They further precipitated the protein from polyoma virus transformed cell line. The authors postulated that this protein may have an important role in the modulation of the transformed state, and it may normally act as a regulator of cellular functions related to growth. Lane and Crawford concluded that it is of prime importance to determine the level of this protein in untransformed cells or normal tissue and to see if it is induced by other carcinogens [214]. The importance of this discovery was not clear early on, since these viruses do not cause human

cancers.

From its humble beginnings, the influence of p_{53} rapidly grew. The number of publications with p_{53} in the leading role increased exponentially every year from 1979. In 1993 p_{53} was named as Science magazines Molecule of the Year[203]. In the editorial Dr Koshland commented that the early detection of p_{53} may lead to treatment by known methods such as surgery, and stressed the importance of basic research - "Basic research does not mean of no practical value"[203]. This is also the aspiration of this piece of research.

Fig13: Cover of Science Dec 1993.

A mutant p_{53} gene synthesises a defective p_{53} protein that lacks the ability to regulate cell proliferation. The p_{53} protein is found in small quantities in normal cells, but in large quantities, 5 to 100-fold increase, in transformed cells both in vivo and vitro. Inactivation of p_{53} is the single most common mutation in human cancer [224]. The presence of p_{53} nuclear reactivity is a predictor of recurrence after definite local therapy i.e. radiotherapy or radical prostatectomy[142]. P_{53} and Ki-67 have been shown to be independent prognostic markers of recurrence [274].

3.11 Function of p_{53} -Guardian of the genome [213].

The wild type p_{53} is capable of forming a homo oligomer, which binds to specific DNA sequences and act as a transcription factor. Insults to cellular DNA, such as exposure to ultraviolet light, or radiation results in an increased levels of the p_{53} protein in normal cells leading to arrest in the G1phase (the growth phase of the cell cycle after mitosis and prior to DNA synthesis), allowing repair of the DNA prior to replication of the DNA in the S-phase of the cell cycle. If DNA repair cannot be accomplished, programmed cell death (apoptosis) may occur. Thus p_{53} is thought to ensure genomic integrity. The p_{53} gene does not directly establish integrity of the genome but acts as a

transcription factor to increase expression of other genes that will initiate the cell cycle arrest and/or apoptosis. Apoptosis is genetically programmed cell death program, which functions as a suicide pathway wherein individual cells kill themselves when triggered to do so. Apoptotic death is distinct from necrotic death.

It has been suggested that p_{53} regulates the transcription of at least two sets of genes; one is responsible for transient cell arrest in G1 and the other controls the initiation of apoptosis. Both of these processes eliminate potential oncogenic mutations, either by DNA repair or by inducing damaged cells to commit suicide.

This response has been lost in many tumour cells as their p_{53} genes have either been mutated or had its activity neutralised through the production of proteins that bind to it and neutralise it. Lack of p_{53} function in tumour cells is thought to be responsible for the observed genomic instability of these cells as manifested by aneuploidy and the ability to undergo gene amplification. However, the p_{53} gene does not seem to be essential for normal development but lack of p_{53} function confers an elevated risk of developing cancer – Li Fraumeni syndrome (see next section).

Initially the p_{53} gene was thought to be an oncogene but later studies showed

that the p_{53} clones that had been studied were in fact mutated versions of the gene, and these mutant alleles have properties which are different from those of the wild type p_{53} gene. Current evidence supports its classification as a tumour suppressor gene.

3.12 Evidence supporting wild type p_{53} as a tumour suppressor gene:

1) The murine wild type p_{53} gene can suppress the transformation of rat embryo fibroblasts in cell culture by known oncogenes e.g. *ras* and adenovirus E1B. The introduction of wild type gene or cDNA into a transformed cell culture stops cell growth at the G1 phase of the cell cycle following mitosis [134].

2) The wild type p_{53} gene is capable of reverting the transformed phenotype of human cancer cells and osteosarcoma cells in an animal model[280].

3) Mutations and allelic loss of the p_{53} gene have been associated with tumours from a wide variety of human organs and tissues, e.g. colon[67], lung[181], oesophagus[70], breast[18], ovary[223], brain[395], and haematopoietic tissue.

4) The Li-Fraumeni familial cancer syndrome[400], characterised by diverse mesenchymal and epithelial neoplasms at multiple sites – breast and adrenocortical carcinomas, sarcomas of soft tissue and bone, brain tumours and leukaemia, is associated with the presence of germ line

mutations in the p_{53} gene[400].

5) Transgenic mice deficient for p_{53} or carrying altered p_{53} gene are significantly more prone to the spontaneous development of a variety of neoplasms[416].

6) Oncoproteins from several DNA tumour viruses e.g. the SV40 large T antigen, the adenovirus EIB, and papilomavirus E6 protein, were found to functionally inactivate p_{53} by binding to or degrading the wild type p_{53} protein[87].

3.13 Mechanism of action of p_{53}.

1) Up regulation of transcription of the bax gene and down regulation of bcl_2[261].

2) Up regulation of IGF-BP3 gene expression. IGF-BP3 blocks IGF induced signalling by binding to IGF. Inhibiting IGF promotes apoptosis[64].

3) Up regulation of FAS/APO-1 gene expression. The cell surface receptor Fas mediates apoptosis [279].

4) Activation of G proteins. Cdc42 is a GTPase, and p_{53} promotes apoptosis through up regulation of cdc42 mRNA and protein, up regulation was followed by cytoplasmic to membrane translocation of this G protein. One action of Cdc42 is phosphorylation and so inactivation of Bcl_2[386].

3.14 Inactivation of p_{53} function

A mutation of p_{53} gene is the most common mechanism of inactivation of p_{53} function [168]. DNA mutation can result from external environmental factors or internal errors in mechanisms involved in nucleic acid metabolism [361]. In the first case, the mutagenic agent determines the nature of the lesion. In the second case, mutations appear to be spontaneous. Analyses of the mutational spectrum of p_{53} in human tumours revealed that the majority of p_{53} mutations in human cancers are missense mutations, giving rise to an altered protein [75]. At least four mutation "hot spots," have been identified in a variety of human neoplasms. Mutations in these hot spot codons account for approximately 30% of all p_{53} mutations.

In addition to mutation, inactivation of p_{53} function can occur by other mechanisms, including binding of p_{53} to another protein or increased degradation of p_{53}. The EIB protein of adenovirus binds to p_{53} and prevents p_{53} activated transcription. The E6 protein of some types of human papilloma virus (HPV) binds to p_{53}, causing p_{53} to be marked for degradation [171]. Thus, HPV infection can lead to increase p_{53} degradation. Only E6 proteins from those strains of HPV that have been strongly associated with anogenital cancers (HPV 16 and HPV 18 are the most common) result in accelerated p_{53} degradation.

Another mechanism by which p_{53} is inactivated is interaction with endogenous cellular proteins. In sarcomas, there is amplification of the MDM2 (mouse double minute) gene, located at chromosome 12q13-14, resulting in high levels of its 90-kD nuclear protein product [291]. Binding of the MDM2 protein to p_{53} is thought to inactivate its transcriptional activity [262]. The binding occurs at a site distinct to the T Ag binding site, and in tumours where there is up regulation of MDM2, mutation of wild type p_{53} is not necessary for transformation [213].

3.15 Detection of p_{53} gene and product

The methods currently used in the detection of p_{53} alterations are outlined below. Different methods have different advantages and disadvantages, which are discussed.

3.16 Immunohistochemical analysis

In 1982 Benchimol et al [23] demonstrated by radio-immununological assay that the p_{53} protein was specifically over-expressed in transformed cells and 'undetectable' in normal cells. Numerous studies have confirmed these observations and proved that p_{53} protein accumulation is a consequence of its stabilisation. Wild type p_{53} (normal) product has a short half-life of about 6-20 min and normally does not accumulate in amounts detectable by conventional immunoprecipitation or immunohistochemical methods [18]. Mutations in the

gene result in a product (mutant p_{53}) with a much longer half-life of up to 6 hours [224]. Therefore, the findings of intense nuclear staining with anti-p_{53} antibody is indicative of a point mutated p_{53} [18]. Early data from direct DNA sequencing confirms that mutations were present in cases that over expressed the protein and were absent in tumours that did not over express the protein [18] [24:146].

Immunostaining studies of tumour cells require antibodies with very high specificity, absence of cross-reactivity with another cellular protein, high affinity for the antigen regardless of the method of tissue fixation. Almost all archival tissue in histopathology departments all over the world is preserved by formaldehyde and embedded in paraffin blocks. This has led to the production of a new panel of monoclonal antibodies (MAbs) specific for human p_{53}, such as DO1, D07 and HR231 [219:407] which are very efficient on this type of material. All these monoclonal antibodies are currently available commercially.

At present, immunohistochemistry is the most widely used technique for assessing p_{53} alterations in human cancers because it is simple and rapid [361]. From the clinical standpoint p_{53} immunohistochemistry appears to be the most practical and useful method for detection of p_{53} alterations in pre-cancerous lesions and carcinomas. This technique allows precise localisation and

identification of the cells that exhibit p_{53} alterations, however it cannot detect the complete loss of p_{53} or non-sense mutations that introduce premature stop codons into the gene[75]. Moreover point mutations in the p_{53} gene are not the only mechanism by which the p_{53} protein can be stabilised. The normal p_{53} protein can be stabilised by the action of viral and cellular oncoproteins. Treatment of cells with DNA-damaging agents (preoperative radio and chemotherapy) increases the level and stability of the p_{53} protein as well [153]. Therefore, interpretation of immunohistochemically positive staining with p_{53} must include consideration of the mechanisms that underlie stabilisation of the p_{53} protein.

A comparative study carried out on 19 archival specimens of colorectal cancer and comparing six commercially available antibodies – Bp53-12(BioGenex), 1801 (Oncogene Science), D07 (Novocastra), 240(Novocastra), CM1 (Novocastra), and Signet (Signet) with results obtained by PCR and double-stranded DNA sequencing demonstrated that D07 gave significantly the best results. When D07 was used with an antigen retrieval system and the cut of for high expression was a labelling index of 30% the sensitivity was 67%, the specificity was 90%, the positive predictive value was 86%, the negative predictive value was 75%, and the efficiency was 79%. If the cut of for the labelling index was lowered to 1% the sensitivity was 89%, the negative predictive value was 86%, specificity was 60%, and the positive predictive

value was 67%, with an efficiency of 74%[13].

3.17 Molecular analysis (DNA sequencing)

DNA sequencing determines the exact mutational event that modified the p_{53} gene. This knowledge is mainly of use in molecular epidemiological analysis. It is generally assumed that a decrease in genetic stability of the genome is involved in the accumulation of a multitude of genetic changes leading to the formation of a neoplastic cell. As mentioned before, the type of p_{53} mutation depends on the event, which affects the DNA (external or internal). Genetic assay is the most accurate technique for identification of p_{53} mutations but it is generally laborious and requires specially equipped molecular laboratories.

3.18 Serological analysis

Several studies have demonstrated the presence of p_{53} auto antibodies in patients with carcinomas [68:91:98:419]. The immune response was found only when p_{53} accumulation was detected in the tumour cell, but mutation or over-expression did not automatically lead to a p_{53} immune response and there was no correlation with the location of the mutation [212:342]. Several laboratories have developed ELISA procedures. Despite this, serological analysis of p_{53} alterations in human cancers is still in its infancy [361]. Progress in this field will be of great importance, because this technique can be easily performed for

routine diagnosis and does not require tumour samples.

3.20 P_{53} and human cancers

Mutations in the p_{53} gene are now regarded as one of the commonest specific genetic changes found in human cancer[75]. More than a half of human malignancies derived from the epithelial, mesenchymal, haematopoietic, and lymphoid tissues, as well as the central nervous system, analysed thus far, have an altered p_{53} gene [17;89;146;168]. This has led to a concentration of efforts to uncover the value of p_{53} as an independent marker for tumour detection and diagnosis, prognosis and progression.

3.21 P_{53} as an independent marker for tumour detection and early diagnosis

Mutated p_{53} protein might be a good target for cancer detection and diagnosis. As discussed before this is primarily due to many missense mutations inducing changes which prolong the half-life of the p_{53} protein allowing immunohistochemical detection. P_{53} positive immunostaining has been observed in pre-cancerous lesions of the colorectum [190;307] stomach [357] bronchus [288;362] larynx [105] oesophagus [25;410] and in head and neck premalignancies[420]. Although p_{53} mutation seems to occur at the earliest clinically detectable stages of the neoplastic process in some cancers, the fraction of tumours with an altered p_{53} gene is generally higher in the late stage of tumours [114;119].

3.22 P_{53} as an independent marker for tumour prognosis

Altered p_{53} function has been examined as potential prognostic factors in a variety of cancers. An ever increasing number of studies are reporting that altered p_{53} is an independent predictive factor of unfavourable prognosis. Aberrant forms of p_{53} protein are correlated with more aggressive tumours, early metastasis and lower 5-year survival rates.

In breast cancer p_{53} over expression (with either demonstrated or presumed mutation) has been associated with late stage, metastatic spread, and low progesterone receptor content [97]. In other studies it was associated with a shortened survival [182;327;336;359;373;390] and shortened metastasis free survival [391]. A consistent picture is now also emerging correlating the loss of p_{53} function with shortened survival in patients with cancers of the colon[67;272;380;427] lung [181;268] oesophagus [70] stomach [118] ovaries [223] and bladder [349] as well as soft tissue sarcomas [108].

3.23 P_{53} as an independent marker for tumour progression

The role of p_{53} mutations in the progression of astrocytomas to glioblastomas [147;358], in the evolution of chronic myelocytic leukaemia to blast crisis [119;408] and in primary as compared with metastatic cervical cancers [92] has been demonstrated. Genetic analysis showed the presence of p_{53} mutant cells in low numbers in primary tumours and the largely mutant composition of the more

advanced tumours as well as their metastases [147:358:408]. In the pathogenesis of colon carcinoma point mutations typically arise during the conversion of a benign adenoma to an invasive adenocarcinoma [114]. The molecular mechanisms that underlie tumour progression are thought to include the clonal expansion of cells that previously acquired a mutation in cancer-related genes. A cell that carries a specific change in a critical gene might acquire a selective growth advantage and become the dominant cell type as the tumour progresses. This hypothesis is also supported by the repeated discovery of the mutant p_{53} preferentially in anaplastic and undifferentiated tumours [106:183:189] e.g. in thyroid carcinomas in which the p_{53} mutants are primarily confined to undifferentiated tumours. The colorectal tumour progression model continues to be the paradigm for all tumour progression.

3.3 P_{53} and Prostate Cancer.

A literature review was carried out using the Medline database. The initial keyword used was p_{53} and the second was prostate neoplasms. For the first keyword a total of 16282 references were detected and for the second 32852. Theses two searches were combined and more manageable 297 references were detected. These were further limited to English language and human and the number of references coned down to 280. The abstracts were reviewed and those that were felt not to be pertinent were omitted, a total of 90 references were reviewed in detail [10;11;19-21;29;32;42;55;56;60;63;65;66;74;75;78; 79;81;100;103;110;112;121;123;139;142;151;161;162;173-176;180;187;193;194;201;209;210;215;220;236;238;238-241;251;254;259;273;274;278;283;295;312;314;315;334;341;364;368;369;371;372;374;375;377;387;389;396;397;399;406;413;423;425;426]

Fifteen years ago the first study linking the inactivation of the p_{53} gene and prostate cancer was published [180]. Since this study there has been an explosion in research activity associated with p_{53} and prostate cancer. In this seminal study by Isaacs et al it was reported that mutations occurred in the p_{53} gene in prostate cancer cell lines and human tissue specimens. Sequence analysis of exons 5-8 of the p_{53} gene reveals that three of five prostate cancer cell lines contain mutations which alter the base sequence of this portion of the gene. One of two primary prostatic cancer specimens examined also contained a mutation in this region. Transfection of the wild-type p_{53} gene versus a mutated p_{53} gene into two cell lines with p_{53} mutations results in reduced

colony formation. Immunocytochemical data indicate that prostate carcinoma cells expressing the transfected wild-type p_{53} gene are growth arrested because they exhibit a reduced level of thymidine incorporation into DNA.

The first paper linking p_{53} and prostate cancer specimens from a patient appeared in 1992 from the group from North Carolina[112]. Using PCR techniques they detected p_{53} mutations in a patient with $T_4N_1M_0$ prostate cancer who had initially been treated with a multimodality combination of radiotherapy and hormonal therapy. The patient 14 years after the initial treatment went on to have a TURP and radical cystoprostatectomy. The areas of the primary tumour and the lymph nodes were examined with PCR techniques. Although the mutations in p_{53} were heterogeneous in the primary tumour, the same mutations were detected both in the primary tumour and the nodal deposits.

3.31 P_{53} in patients who have undergone surgical treatment.

Radical prostatectomy has become the main stay for the surgical management of localised prostate cancer; however it is only successful if the patient is cured. P_{53} has been advocated as a predictor of outcome. One of the earliest studies of the incidence of p_{53} in patients with prostate cancer consisted of 150 patients. The study group consisted of 88 men who had had a radical prostatectomy, 52 cases had a TUR, and 10 cases had a TUR for palliation of

recurrent disease. P_{53} was detected using immunohistochemistry in 17 of the 140 primary tumours, and in two patients with recurrence[42]. It is noteworthy that an antigen retrieval system was not used in this earlier study. Immunostaining was significantly related to stage. The immunhistochemical findings were confirmed in 14 cases by DNA amplification and screening between exons 4-9. 20 negatively staining cases were used as controls and no mutations were detected in these cases. The authors used the fact that single strand conformational polymorphism may not be 100% sensitive, and p_{53} accumulation may occur without p_{53} mutation as an explanation for the discrepancy in the results obtained between immunohistochemistry and single strand conformational polymorphism. The authors also commented upon the fact that compared to other tumours – breast, lung, colon, and oesophagus, the frequency of p_{53} mutation and accumulation was rather low[42].

In a retrospective study of 49 patients with clinically localised (T_{1-2} N_x M_0 , PSA < 20, bone scan negative) moderately differentiated (Gleason score 5-7) carcinoma of the prostate treated with radical prostatectomy and with a follow up of at least 5 years, Yang et al [425] showed that p_{53} determined by immunohistochemical was present in 10/16 patients which had a recurrence compared to 7/33 in the non-recurrent group. P_{53} was significantly associated with cancer recurrence. It is interesting to note that in this report 22 out of the 49 patients that had extra capsular disease and the incidence of nodal

involvement is not mentioned.

In a study of 93 patients from Memorial Sloan-Kettering Cancer Centre, 19/93 (20%) demonstrated nuclear immunostaining [11]. Of the 93 tumours, 48 were primary tumours, 29 were lymph nodes involved with tumour, and 16 were specimens from patients who had presented with pathological fractures. Of the 48 primary tumours, 9 were organ confined, 18 had seminal vesicle involvement, at the time of the operation 11 had lymph node involvement, and 10 were from patients with disseminated hormone refractory disease. Apart from the 10 patients with metastatic disease who had undergone palliative TURPs, the remaining 38 primary tumours were from patients who had under gone radical prostatectomy. 9 out of the 48 primary tumours stained positively for p_{53}, of these 9, 8 had Gleason scores above 8. Only one of the primary tumours with a Gleason score of less than 7 was observed to accumulate p_{53}. None of the specimens with organ confined disease stained positively for p_{53}, but 1 out of the 18 with seminal vesicle involvement, and 2 out of the 11 patients with pelvic lymph node metastasis stained positively for p_{53}. Eight out of the 16 bone specimens compared to two out of the 29 lymph nodes stained positively for p_{53}. Therefore p_{53} accumulation significantly correlated with high Gleason score, stage, hormone refractory disease, and was more common in bone metastasis than lymph node metastasis. A study of 100 consecutive radical prostatectomy specimens confirmed the low incidence of p_{53} mutations

in localised carcinoma i.e. one in 67 cases, compared to 5 out of 33 cases in locally invasive carcinoma [273]. This study also demonstrated a good correlation between immunohistochemically detected p_{53} over expression compared to p_{53} mutations detected by polymerase chain reaction amplification and single strand conformation polymorphism. The DO-1 antibody detected 5 out of 6 mutations in p_{53} and an additional mutation in exon 6 was detected by molecular biology [273].

A further study of 73 radical prostatectomy specimens demonstrated that 34 specimens stained positively for p_{53} [162]. It is interesting to note that only 28 out of the 73 prostatectomy specimens were in fact organ confined i.e. 39%. 24 out of the 28 pT_2 or less lesions were p_{53} negative, thus staining for p_{53} correlated with high stage. In addition p_{53} staining correlated with increased PSA, Gleason score and increased tumour volume. This correlation is not surprising since high stage disease is associated with increased PSA, high Gleason score and high tumour volume. Follow up was not an aspect of this study and this paper did not comment upon whether p_{53} staining was an independent marker for recurrence. A further study of 137 consecutive prostate specimens obtained by radical prostatectomy detected 11 specimens (8%) with p_{53} over expression, ten out of the 11 specimens were pT3, nine out of the 11 were high grade i.e. Gleason score >7. P_{53} over expression was associated with increased Ki-67 labelling index implying rapid tumour

proliferation [60].

A study of 85 prostate carcinoma specimens obtained from a mixture of radical prostatectomy specimens and TURP specimens analysed by immunohistochemistry and DNA analysis demonstrated only 3 specimens with aberrant p_{53} expression i.e. 3.5% [406]. No information was given regarding the stage of the disease; the Gleason score of the specimens was 6, 7, and 8. The authors concluded that the presence of aberrant p_{53} appears to be an infrequent event. The infrequency of p_{53} in localised prostate cancer was further confirmed on a study of tissue obtained from 37 patients with localised carcinoma of the prostate and subsequently treated with external beam radiotherapy, only one of the 37 specimens stained positively for p_{53} [151]. This study went on to compare immunohistochemistry with PCR using both positive and negative samples and found complete concordance. These authors concluded that the presence of p_{53} mutation was a late step in the progression of prostate cancer and was a late step in the progression of prostate cancer and associated with advanced disease, dedifferentiation, and the acquisition of androgen independence.

The studies so far mentioned in this section have largely concentrated upon detecting the presence of p_{53}, and correlating it with known markers of poor outcome, i.e. high Gleason score, high tumour volume/stage. The group from

Walter Reed Army Medical Centre, Washington, then published the outcome of their series of radical prostatectomy operations [19:20:274]. The series consisted of 175 patients who had had radical prostatectomy over a seven-year period and who they had archival material available on. Using immunohistochemistry 114 of the 175 patients (65%) were positive for p_{53}, a much higher proportion than previously detected. Expression correlated with higher stage, Gleason score, and nuclear grade. P_{53} positivitey did correlate with a higher incidence of recurrence, i.e. 51.1% in p_{53} positive patients versus 22% in p_{53} negative patients. This was an independent prognostic marker [19]. Over expression of bcl_2 was detected in 47 patients (26.9%), the combination of p_{53} positive and bcl_2 positive was highly significant at predicting recurrence when compared to p_{53} negative and bcl_2 negative patients. Similar results have been reported by others, in a series of 40 radical prostatectomy specimens, 32 (80%) stained for p_{53} and of these 20 (62.5%) progressed, none of the p_{53} negative patients progressed. P_{53} immunoreactivity was an independent predictor of recurrence [374]. In a British series of 76 radical prostatectomies carried out by the Bristol group with a mean follow-up of 38 months, aberrant p_{53} was detected in 39 (57%) of patients. 19 of these patients had recurrence of prostate cancer, where as in the group with normal expression of p_{53} only three out of 23 patients had evidence of recurrent disease. Therefore the authors concluded that aberrant p_{53} does predict recurrence [55]. In a further series of 76 radical prostatectomies, aberrant p_{53} was detected in 18 patients (20% positive

staining) and this significantly correlated with poor survival in a multivariate analysis [209].

A heterogeneous series of patients treated with radical prostatectomy, some having had LHRH therapy preoperatively, from the Memorial Sloan-Kettering demonstrated a strong link between p_{53} over expression and recurrence [295]. However they also found that some tumours expressed p_{21}. There was no relationship between p_{21} and p_{53} status, this is interesting since p_{21} is thought to be a downstream messenger of p_{53}, and one would expect only patients with wild type p_{53} to have expression of p_{21}. The authors postulated that p_{21} may also be activated by a p_{53} independent pathway [295].

A study from New South Wales carried out on 263 consecutive radical prostatectomy attempted to quantify the relationship between p_{53} and outcome after surgery[312]. The authors compared the number of cell with aberrant p_{53} with outcome, and also compared clustering of p_{53} i.e. the presence of a cluster of p_{53} positive tumour cells within a 200 x magnification field, with outcome. They concluded that their data supported the relationship between outcome and p_{53} immunohistochemistry at thresholds of no nuclear accumulation versus any accumulation; and the presence of clusters of p_{53} positive cells versus their absence; and between >20% positive cells and <20%staining. In predicting early death from prostate cancer p_{53} positivity in >20% of nuclei defines a

group of patients with highly aggressive disease, all six of the patients that died during the follow up period of the study (4.9 to 123 months, mean 55.5months) fell in to this group [312].

A recent study investigated the usefulness of p_{53} in locally advanced prostate cancer [220]. This study group consisted of 72 patients with a median follow up of 5.4 years (range .5 to 6.4). Biochemical failure, local failure, or systemic failure occurred in 29 of the patients. The group compared the usefulness of p_{53} positive staining at the 10% level with Gleason score, PSA, pathological stage, volume of extraprostatic tumour extension, and total volume of tumour. Using a multivariate analysis, which contained p_{53}, only p_{53} predicted outcome, and when it was added to the statistical model for predicting outcome it improved the predictive ability of the model[220].

Other studies have not shown p_{53} to be so useful. A study evaluating the role of p_{53}, Ki-67, and apoptotic index in predicting recurrence in a series of 47 patients demonstrated that 16 of the specimens had clustered immunopositivity for p_{53}. 17 of the 47 patients had evidence of disease recurrence at 5 years and the conclusion of these authors was that although both p_{53} and apoptotic index may predict recurrence using a multivariate analysis only the apoptotic index reaches significance in the final logistic regression [368]. A further study of 208 consecutive radical prostatectomy specimens, 75 with localised intracapsular

disease, 123 extra capsular locally invasive, and 8 of these had lymph node involvement. P_{53} at the >5% level was detected in 79 specimens, and although this did correlate with tumour volume, p_{53} expression levels were not associated with differences in recurrence free survival [194]. A study of 134 patients also showed that p_{53} was not helpful; this report did not indicate the method of treatment [397].

3.32 P_{53} and watchful waiting

The Scandinavian countries have adopted watchful waiting as their mainstay for the management of asymptomatic carcinoma of the prostate. The rational for this has been discussed previously (see section 1.66). In a study of 186 patients diagnosed by TURP and treated with watchful waiting 163 patients died, 79 from prostate cancer, during the follow up. Nineteen patients stained for aberrant p_{53}, the mean survival for p_{53} positive patients was 52 months and for p_{53} negative patients it was 123 months. There was a significant correlation between tumor stage, tumor grade, metastasis, age and p_{53} staining. However in a Cox multiple regression analysis of tumor stage, tumor grade, metastasis, age and p_{53}, p_{53} lost its significance. The authors concluded that the prognostic value of p_{53} appears to depend upon its association with advanced stage and high grade, and they did not feel it would be a good prognostic marker in early prostate cancer[369]

A further study from Scandinavia of 221 men diagnosed with carcinoma at TURP and followed up for a median of 15 years showed that all tumors expressed some degree of staining for p_{53}. There was a significant association between p_{53} staining, local tumor stage and grade. The patients that were highly immunoreactive for p_{53} did significantly worse than the patients with low immunoreactivity for p_{53} [43].

3.33 P_{53} and radiotherapy.

Radiation causes cellular DNA damage which, if irreparable can lead to apoptosis in a cell expressing wild type p_{53}. Cells with an aberrant p_{53} can not enter cell cycle arrest at the G1/S or G_2/M checkpoints, as would cells expressing the wild type p_{53} gene. In a series of 33 patients who had failed radiotherapy and had pre and post radiotherapy specimens no significant difference in p_{53} immunostaining was found between the failures (67%) and the successes (75%) of radiation therapy [173]. However, the sample size was small and the follow up period was short at 3 years. A further study of 38 patients also failed to show any relationship between p_{53} over expression and recurrence [315].

A large study was commissioned under the auspices of the Radiation Therapy Oncology Group. The protocol, 8610, consisted of cytoreduction pre and peri radiotherapy for locally advanced prostate cancer. One hundred and twenty

nine patients were analysed in this sub set analysis with a median follow up of 5 years (range 1.17 – 7.14 years). 23 patients stained for aberrant p_{53} with > 20% positive nuclei, 12 in the radiotherapy alone arm and 11 in the radiotherapy plus cytoreduction group. Using Cox regression analysis abnormal p_{53} expression was an independent prognostic indicator for significantly shortened survival, increased incidence of distant metastases, and a reduced progression free survival [139].

A study of 55 patients with recurrent disease despite radiotherapy, who then underwent salvage radical prostatectomy, p_{53} staining was detected in 50 specimens[79]. Over expression of p_{53} was associated with an increased proliferative activity as measured by Ki-67 labelling index. It is not clear from this study whether the over expression of p_{53} developed overtime or was always present. Additionally radiotherapy can lead to stabilisation of the wild type p_{53} and so this may be detected by immunohistochemistry. An older study of 18 radiotherapy failures patients also showed a high level of p_{53} accumulation (72% - 13 patients) [310]. In this study group the authors had pre radiotherapy tissue specimens of 10 patients and in these specimens there was also a high level of p_{53} expression, implying longstanding p_{53} alteration. The authors also tested the validity of p_{53} immunoreactivity with PAb 1801 in ten post irradiation patients, five with positive staining. Using single strand conformational polymorphism they could only demonstrate abnormalities

between exons 5 to 8 in three out of the five p_{53} positive staining patients. None of the negative staining specimens demonstrated any abnormalities. The authors attributed this discrepancy to less than 100% sensitivity of single strand conformational polymorphism, false positive staining with immunohistochemistry, or the p_{53} mutations may lie outside exons 5 to 8 [310].

The alteration in the phenotype and the genotype of recurrent tumours after definitive therapy was investigated in 32 patients [142]. 18 patients had recurrence after radical prostatectomy, and 13 had recurrence after radiotherapy. In both groups of patients there was a significant increase in the Ki-67 labelling index, and p_{53} nuclear reactivity in tissue obtained from recurrences when compared with primary lesion. The nuclear reactivity was of the clustered type, which may represent a more aggressive phenotype [425]. There was also an increase in the Gleason scores of the recurrent lesions in the radiotherapy group. This would suggest that a more aggressive phenotype. The authors suggested a number of explanations for this; tumour cells are genetically unstable and therefore the first cells to relapse are the ones with a selective growth advantage. An alternative explanation may be by removing the primary tumour, a growth inhibitor is removed. There is therefore growth acceleration of the residual tissue as is the case in the Lewis lung carcinoma model [289].

3.34 P_{53} and pre-treatment biopsies.

The ability to detect preoperatively those patients that would benefit from radical surgery would be a considerable aid to the surgeons. In a series of 76 patients who had undergone radical prostatectomy and had preoperative TRUS guided biopsies it was shown that the risk of relapse was 24% higher if aberrant p_{53} was detected on the pre-operative biopsies. Using a multivariate analysis the presence of aberrant p_{53} and Gleason score > 7 was shown to be independent predictors of relapse in patients undergoing radical prostatectomy[55].

A study comparing biopsies with 106 radical prostatectomy specimens that had neoadjuvant androgen ablation prior to definitive surgery did not show a correlation between preoperative expression of p_{53} and outcome [74]. In this study, with respect to radical prostatectomy specimens, the aberrant p_{53} was detected in 32 cases prior to antigen retrieval and in 77 cases after antigen retrieval. Only 77 biopsies were available for analysis and after antigen retrieval only eight were positive. The expression of aberrant p_{53} in radical prostatectomy specimens correlated with poor outcome, however in a multivariate analysis this was not an independent prognostic factor. Biopsy expression of p_{53} did not relate to outcome [74]. The increase in the levels of p_{53} may be explained by the effect of androgen ablation on the expression of p_{53}.

Prostatic Intraepithelial Neoplasia and Prostate Cancer.

A retrospective review of the pre-treatment biopsies of 49 patients who underwent definitive radiotherapy for the treatment of localised carcinoma was carried out. A surrogate end point for relapse was used; this was a PSA nadir of 1.0ng/ml. The biopsies of 17 patients stained positively for p_{53}, of these patients 15 (88.2%) did not reach a PSA nadir of 1.0. 32 patients stained negatively for p_{53}, of these 23 reached the surrogate end point. Logistic regression analysis showed that p_{53} was an independent predictor of relapse after definitive radiotherapy [341]. The results of this study could be interpreted as showing that increased p_{53} is common in patients with advanced possibly extraprostatic disease not amenable to radiotherapy.

A comparison of pre-treatment biopsies and survival was carried out on 60 patients [371]. This was a retrospective study, with the majority of patients not receiving any pre-treatment hormonal manipulation. The group of patients consisted of 36 patients with T_2 or below disease and 18 patients with stage T3-T4 disease. Six patients were Tx, i.e. not staged; the local staging was carried out with digital rectal examination. 15 patients underwent lymph node dissection, and five of these were positive. 59 patients had had a bone scan which was negative. P_{53} immunostaining was carried out with an antigen retrieval system, overall positive staining occurred in 15 tumours, surprisingly and in contrast to other studies a greater proportion of lower stage tumours stained positively. The survival of the p_{53} positive group appeared to be better

than the p_{53} negative group. The authors concluded that no survival disadvantage was seen in patients with p_{53} immunoreactive tumours, implying aberrant p_{53} does not confer radio resistance and that alternative pathways to apoptosis may be more important. This study is incomplete in many ways. There is no mention of PSA, and no imaging of the lymph nodes was carried. It is not incomprehensible to suspect that many of these patients may have advanced disease since in the 15 patients suitable for radical surgery a third had disease beyond the prostate and mean survival of the patients was only 75 months (range 8.5 – 172). [371].

3.35 P_{53} and metastatic prostate cancer

A study of ten lymph node specimens with prostate cancer showed that only one stained positively for p_{53} [406]. A further study of 56 patients with metastatic lymph node disease and a median follow up of 3.8 years was carried out [238]. In this study 26 patients had material available from both the primary tumour and lymph nodes, a further 15 had material available from the primary tumours only, and 15 patients had only lymph node samples. P_{53} was detected in 5 primary tumours, and in 7 lymph node specimens. The median Ki-67 labelling index was 8.4% in the primary tumours and 8.7% in the lymph nodes. The median Ki-67 labelling index was significantly higher in the p_{53} positive patients. Using a multivariate analysis the Ki-67 labelling index and Gleason scores of the primary tumour were independent prognostic factors, in patients

with positively staining lymph nodes the presence of bone metastasis was also a prognostic factor [238]. Although patients staining positively for p_{53} and bcl_2 tended to have a poorer outcome this did not reach significance [238]. A study of 36 primary prostate cancers, 17 patients with nodal involvement, and 14 cases of bone metastasis, demonstrated a greater proportion of the specimens obtained from metastasis showed aberrant staining for p_{53}; i.e. only ten of the primary tumours, ten of the lymph nodes with metastasis, and six of the bone metastasis [174]. Interestingly there was no correlation between p_{53} expression in the primary tumour and metastasis.

The difference in p_{53} staining between treatment naïve, metastatic and hormone refractory prostate cancer was investigated in 61 patients[161]. The 61 patients consisted of 27 patients with recurrence after either radiotherapy or hormonal intervention or both and tissue was obtained by TURP and in one case by prostate biopsy; 8 patients with metastatic lymph node disease and tissue was obtained from the lymph nodes; and 27 primary untreated tumours and tissue was prostate biopsies. The authors demonstrated an increase in p_{53} in the hormone refractory group i.e. 20 out of the 26 cases (76%) stained positively for p_{53} compared to 4 out of the 8 (50%) metastatic, and 6 out of 27 (22%) in the untreated group. The authors concluded that this study demonstrated an increase in p_{53} from untreated primary tumour to hormone refractory disease[161]. However there are several points in this paper, which

are not that clear. The group of patients with refractory disease was rather heterogeneous, and it is not clear whether all the patients in the relapse group actually relapsed using PSA criteria or needed a TURP for non-malignant outlet obstruction. The abstract of the paper claims that 94% of the patients in this group were p_{53} positive, where as in the body of the paper only 76% i.e.20 out of 26 stain positively for p_{53}. In addition it is worth noting that five patients in the relapsed group had cytoplasmic staining for p_{53}, the authors graded these as positive[161].

A study of bone marrow biopsies obtained from the iliac crests of 43 patients with hormone refractory metastatic carcinoma of the prostate showed that 22 (51%) exhibited immunohistochemical positivity for p_{53} [245]. Eight cases exhibited positive staining for bcl_2 and p_{53}, and six cases were positive for just bcl_2. The expression of either of these proteins did not influence outcome and so it was concluded that these genetic mutations are not essential for the fatal progression of prostate cancer. There must be multiple pathways of tumour progression in prostate cancer [245].

3.36 The effect of castration on p_{53}.

In a retrospective study, Stattin et al studied the effect of castration on 28 patients with carcinoma of the prostate [372]. The 28 patients consisted of 15 responders and 13 non-responders, 3 patients were initially stained for p_{53} and

further 3 responders stained after LHRH therapy. P_{53} immunoreactivity did not correlate with outcome after castration [372].

Increased p_{53} was also detected in-patients who had had neoadjuvant LHRH therapy prior to radical prostatectomy. All the patients in this cohort who had expression of p_{53} relapsed. The authors concluded that the finding of an aberrant p_{53} could be due to the advanced stage at which the patients presented and were selected for treatment using neoadjuvant LHRH, since an altered p_{53} may lead to resistance to castration induced apoptosis and so disease relapse. Therefore the presence of an aberrant p_{53} may be an early event in the evolution of hormone refractory disease in clinically localised prostate cancer [295]. This relationship between aberrant p_{53} after neoadjuvant therapy and relapse was further commented upon, when 19 out of 21 patients with p_{53} clustering relapsed within 36 months of definitive treatment [312].

This relationship between the presence of aberrant p_{53} detected immunohistochemically after LHRH treatment was further strengthened in a subset analysis of 263 radical prostatectomies carried out in New South Wales in Australia[312]. 39 patients in this cohort of 263 had received neoadjuvant LHRH treatment prior to definitive surgery. 23 patients in this group of 39 had relapsed. A total of 21 patients were p_{53} cluster positive, and 90% (19 patients) of the cluster positive patients relapsed. P_{53} cluster status was the

strongest predictor of recurrence in a multivariate analysis comparing p_{53}, pretreatment PSA, Gleason score, and pathological stage [312].

In a study of 26 patients with prostate cancer that had relapsed despite first line therapy – radiotherapy or hormonal or both, a total of 20 patients demonstrated p_{53} staining and in 4 of these the staining was cytoplasmic [161]. The significance of cytoplasmic staining of a nuclear protein is at present unclear. However the authors counted these tumors as p_{53} positive.

The relationship between aberrant p_{53} expression and androgen receptor gene amplification was studied in 30 patients with hormone refractory disease. Comparing primary untreated tumours with hormone refractory specimens obtained from the same patient, the immunostaining for p_{53} increased from 17% to 40% respectively. The tumours with aberrant p_{53} expression were more likely to have androgen receptor gene amplification, suggesting that tumours with an aberration in p_{53} were more likely to become hormone refractory and so survive in lower androgen concentrations[201].

3.37 Site of p_{53} mutations

Micro dissected DNA selectively extracted from paraffin-embedded sections of prostate cancer and PIN has shown a high frequency of p_{53} mutations in the lesions dissected from the peripheral zone lesions when compared to those that

were dissected from the transition zones. For the cancer lesions the incidence was 33.3% in the peripheral zone versus 4% in the transition zone, and for PIN the incidence was 14.7% and 5.6% respectively. In areas of benign tissue adjacent to the PIN or cancer there were mutations detected in p_{53}. The point mutations detected in PIN and cancer were not identical the authors attributed this to the polyclonality of prostate cancer. In addition the authors found a higher incidence of p_{53} mutation in T3 carcinomas of the prostate when compared to T_2 lesions. The authors conclude by stating that their findings indicate that that the p_{53} gene mutations are involved in prostatic carcinogenesis and explain why the non-transition zone is the predominant site of cancer[377].

3.38 Racial differences

A heterogeneous group of 62 archival samples of prostate tumor obtained from Asians of non-Japanese origin were examined [81]. The specimens were obtained by TURP and the patients were staged with a CT scan of the abdomen and pelvis, only 2 patients' expressed p_{53}, both of these patients had metastatic disease. The authors concluded that the expression of p_{53} mutation in Asian cohorts is significantly lower.

Table 6 (cont.): The incidence of p_{53} in patients with prostate cancer.

[278]	1994	Rad Pros tumour (28), Rad Pros PIN (16) metastatic primary (16), lymph nodes (18).	78	28	?	16+18	IHC	2/28 localised carcinoma p53+, 1/16 PIN p53+, 9/16 metastatic p53+, 10/18 lymph nodes p53 +.	Increasing p53 in pts with mets.
[32]	1994	Microdisection of benign lesions close to malignant tissue	21	6	12	3	PCR SSCP	1/21 benign lesion close to Gleason 7 lesion showed alteration in p53.	P53 alteration may be an early change in the genesis of prostate cancer.
[161]	1995	TURP + Biopsies	61	?0	26 +27	8	IHC PCR	6/27 treatment naïve p53+, 20/26 androgen refractory p53+, 4/8 metastatic were p53+.	Progression in p53 alteration from untreated to hormone refractory disease.
[151]	1995	TURP + biopsies.	37	26	11	0	IHC PCR	1/37 p53 + P53 positive on IHC confirmed by PCR, 7 negative patients had PCR, no p53 mutation.	IHC is reliable, and correlates well with PCR. The authors concluded p53 mutations detected by IHC are a late step in the progression of prostate cancer and are associated with advanced disease, dedifferentiation, and the acquisition of androgen independence.
[310]	1996	Salvage prostatectomy (15) or TURP (3) for relapse post DXT.	18	?	?	?	IHC + PCR	13/18 (72%) + in relapse group, 20 % + in control (Non irradiated advanced Ca P)	P 53 aberration common in patients with relapse post DXT.

Table 6: The incidence of p_{53} in patients with prostate cancer.

Ref.	Year of pub	Type of specimen	No of cases	No with intracapsular disease	No with extra capsular disease.	No with metastasis	Method	Results of P53 staining	Conclusion
[406]	1994	85 prostate (Rad pros + biopsies)+ 10 lymph nodes	85 + 10	?	?	10	IHC + Polymerase chain reaction – single stranded polymorphism (PCR-SSCP)	3/85 p53 + in primary prostate cancer and 1/10 p53 + in lymph nodes.	P53 mutations appear to be of low frequency in prostate cancer.
[162]	1994	Rad pros	73	28	45		IHC	4/28 localised p53 + 31/45 extracapsular p53 +	P53 correlated with more advanced tumour stages, with higher Gleason grades, and with larger total and high-grade tumour volumes.
[11]	1994	Radpros, TURP, Primary, lymph nodes, Mets	93 (48 prostates 29 lymph nodes, 16 bone mets).	9	18	11 rad pros in pts with lymph node involvement10 TURP 29 lymph node 16 Bone mets	IHC	0/9 p53+ in localised 1/18 p53+ in extra capsular 2/11 p53+in pts with lymph node+ 6/10 p53+ in hormone refrac CaP 2/29 p53+ in lymph node met 8/16p53+ in bone mets.	P53 correlates with high grade, hormone refractory tumours, and bone mets.

Table 6 (cont.): The incidence of p_{53} in patients with prostate cancer.

[423]	1997	Rad Pros. TURP. Pros Biopsy	36	22	?	14	IHC	5/22 (23%) p53 positive localised. 1/14 (7%) metastatic positive.	Follow up only 12 months. no relationship between outcome and p53 status
[273]	1997	Radical prostatectomy	100	67	33	29	IHC PCR-SSCP	1/67 localised p53 + 5/33 extra capsular p53 + 5/6 mutations detected by IHC 6/6 detected by PCR - SSCP.	P53 mutations are a rare late event in prostate cancer.
[66]	1997	Biopsy TURP	40	15	24	21		T_{1-2}=5/15 p53 + T_{3-4}=14/24 p53 + 17/19 p53+ pts progressed 12/21 p53 - pts progressed	P53 positive tumours higher grade and quicker progression. No correlation between p53 and p21 or waf1.
[397]	1998	Not Stated	134	51	27	56	IHC	11/51 p53+ Localised 7/27 p53+ Extra capsular 15/56 p53+ With extra prostatic disease.	P53 did not predict outcome (Mode of treatment not stated)
[79]	1999	Salvage Radical prostatectomy in post-DXT relapse.	55	?	?	?	IHC	50/55 P53 +	High incidence of p53+ in patients who relapsed post DXT.
[81]	2000	62 TURP	62	13	4	36	IHC PCR	2 p53+	Lower p53 expression in Asians
[356]	2004	Rad Prost	98	?	?	?	IHC	55/98 Positive by IHC	No prognostic data. but compared IHC with PCR. IHC generated 27/55 false positive. and 15/42 false negative.

Table 7: The relationship between p_{53} watchful waiting

Ref.	Year of pub	Type of specimen	No of cases	No with intracapsular disease	No with extra capsular disease.	No with metastasis	Method	Results of P53 staining	Conclusion
[369]	1996	TURP	186	159	27	23	IHC	T0 = 6% M0 = 9% T1 = 13% M1 = 22% T2 = 10% Mx = 14% T3 = 25% T4 = 38%	P53 correlates with stage, grade, metastasis, but in multiple regression analysis it is not an independent predictor.
[43]	2000	TURP	221	131	90	60	IHC	P53 staining correlated with stage.	P53 was an independent adverse prognostic indicator.

Table 8: The relationship between p_{53} and outcome in patients who have had radical prostatectomy.

Ref.	Year of pub	Type of specimen	No of cases	No localised	No locally invasive	No lymph nodes/mets	Method	Results	Conclusion.
[42]	1993	Rad Pros +TURP	150	38	36	69	IHC + PCR	Stage 0 1/12 p53 + Stage 1 0/26 p53 + Stage 2 2/36 p53 + Stage 3 8/36 p53 + Stage 4 8/33 p53 + Unstaged 0/7 p53 + {Treatment was radical prostatectomy (88), TURP (52), hormonal manipulation (10)}	19/150 p53 + P53 + related to stage.
[19]	1995	Rad Pros	139	57	81	1	IHC	28/57 localised p53 + 56/81 extracapsular p 53 +	P53 was an independent marker for progression.
[425]	1996	Rad Prost	49	29	20	?		10/16 with recurrence, compared to 7/33 in non-recurrent group.	P53 staining associated with recurrence.
[274]	1996	Rad Pros	162 mean follow up 4.5yrs	62	99	?	IHC	38/62 localised p53 + 73/99 extraapsular p53 +	In a multivariate analysis p53 and Bcl-2 independent prognostic markers. P53 + = 112 (69.1%), bcl-2 = 44 (27.2), Ki-67 = 62 (38.3%)
[20]	1996	Rad Pros	175pts with mean fu 4.6yrs	174	1	?	IHC	113/174 localised p53 + 1/1 extracapsular p 53 +	In a multivariate analysis p53 and Bcl-2 independent prognostic markers. Bcl2+ = 26.9%, p53+ = 65%, multivariate analysis p53 and bcl2 independent predictors of disease.

Table 8 (cont.): The relationship between p_{53} and outcome in patients who have had radical prostatectomy.

[60]	1996	Rad Pros	137	44	93	34	IHC	1/44 localised p53 + 10/93 extra capsular p53 + 3/34 node + pts were p53 + in the primary tumour.	P53 + tended to worse prognosis but not significance. Bcl2 pT3 (31%) > pT2 (5%) significant. Combined analysis of Ki67, LI, and Bcl-2 allowed the distinction of three groups with different clinical outcome. Prognosis was best in Bcl-2-negative tumours with low Ki67 LI, worst in Bcl-2-positive tumours with high Ki67 LI.
[374]	1996	Rad Pros	40	0	40	?	IHC	32/40 p53 +	20/32 p53 + progressed. P53 was an independent marker for progression.
[209]	1998	Rad Pros	76	42	34	6	IHC	18 >20% p53 +	>20% p53 + only variable associated with decreased long-term survival in multivariate analysis.
[194]	1998	Rad Pros	208	75	123	8	IHC	79 p53 +	P53 and Rb did not predict recurrence, but p53 did correlate with tumour volume, MIB-1 and Bcl-2 correlated with progression.
[142]	1998	Rad Pros	18	11	7	5	IHC	1/18 Primary p53 + 7/18 Recurrent p53+	Increased p53 in recurrences, genotype of recurrence is more aggressive.
[368]	1998	Rad prost	47	47	0	0	IHC	10/17 p53+ in relapse group @ 5yrs 6/30 p53+ in non-relapse group @ 5 yr.	In multivariate analysis only apoptotic index and clustered p53 added info, in regression model only AI reached stat sig.

Table 8 (cont.): The relationship between p_{53} and outcome in patients who have had radical prostatectomy.

[55]	1999	Rad Prost + pre-op biopsies.	76	37	39	?	IHC	39 p53+	P53 predicted recurrence. Aberrant p53 led to a 24% higher risk of relapse. P53 expression was more frequently observed in biopsy specimens? difference in fixation? difference in tumour due to multifocality.
[295]	1999	Rad Prost	86	51	35	?	IHC	6/86 >20% p53 +	P53 +ve relapsed quicker. No correlation between p53 + and preop PSA, tumour stage, or grade. Also stain for mdm2 and p21 which was positive in 50% of cases and postulated that p21 activation due to an alternative mechanism other than p53 activation.
[312]	2000	Rad Pros	263	114	144	5	IHC	132 (50.2%) (nuclear clustering) >0 to <2% 33 (12.2%) >2 to <5% 35 (13.3%) >20% 34 (12.9%)	P53+ve (Clustering or nuclear accumulation) was an independent predictors of relapse, as was pre treatment PSA, tumour stage, tumour grade, and lymph node status. In pts treated with preop LHRH p53 clustering predicted relapse.
[220]	2000	Rad Pros in locally advanced disease.	72	0	70	23	IHC	28 >10% p53 +	P53+ predicted biochemical, local, & systemic failure.

Table 8 (cont.): The relationship between p_{53} and outcome in patients who have had radical prostatectomy.

[74]	2000	Rad Pros Biopsies after neoadjuvant treatment.	106	57	31	17	IHC	77/106 p53 + 8/77 biopsies p53 +	P53+ increased after hormonal Tx, and correlated with grade, stage, biochemical progression and short survival. P53+ from pre LHRH biopsies did not show any correlation.
[356]	2004	Rad Prost	98	?	?	?	IHC	55/98 Positive by IHC	No prognostic data, but compared IHC with PCR. IHC generated 27/55 false positive, and 15/42 false negative.
[177]	2005	Rad Pros	52	14	38	0	IHC	20 p53 +	13 p53+ relapsed, 7 p53- relapsed (P<0.0001). p53+

Table 9: The relationship between p_{53} and outcome in patients who have had radiotherapy.

Ref.	Year of pub	Type of specimen	No of cases	No localised	No locally invasive	No with lymph nodes/mets	Method	Results	Conclusion.
[371]	1996	Pre-DXT Biopsies.	60	36	18	5	IHC	Localised = 12/36 p53+. Locally invasive = 3/18 p53+	P53 correlated with stage, not grade. P53 did not predict relapse.
[139]	1997	Pre-DXT Biopsies	129		129		IHC	23/129 (18%) with p53+ >20%. 6/23 p53+ progressed 27/106 p53- progressed 12/23 p53+ died at 4 yr. 43/106 p53- died at 4 yr.	P53 + prognostic indicator for survival, decreased progression free survival, decreased overall survival and distant metastasis. Independent of Gleason score and stage.
[142]	1998	Pre-DXT biopsies & post-DXT biopsies.	13			13	IHC	Primary p53 + = 1/13 (8%) Recurrent p53 + = 7/13 (54%)	Genotype of recurrence is more aggressive than primary lesion.
[173]	1998	Pre-DXT biopsies & post-DXT biopsies.	33 pt. who relapsed after DXT	?	?	?	IHC	P53 + pre DXT = 24/33 P53 + post DXT = 20/33	P53 did not increase post DXT, and could not predict DXT failures.
[341]	1999	Biopsies	49	49			IHC	17/49 p53+ 32/49 p53-	15/17 p53+ did not reach PSA nadir of 1.0 23/32 p53- did reach PSA nadir of 1.0. Pre DXT p53 status predicts response to DXT.

Table 10: P_{53} in patients treated with hormonal manipulation.

Ref.	Year of pub	Type of specimen	No of cases	No localised	No locally invasive	No mets	Method	P53	
[405]	1992	TURP = 44 Biopsy= 75 Open Spec = 18	137	51	82	36	IHC	T1 + T2 =2 % M0 = 2% T3 = 2% M1 = 11% T4 = 12%	Pts treated with hormonal manipulation (n=97), DXT, and surgery. High level staining correlates with early relapse, poor survival, and high grade.
[372]	1998	Biopsies before & after LHRH	28	7	21	0	IHC	3 (6 after LHRH) p53 +ve.	P53+ve could not predict response to LHRH treatment, but increased in responders.
[241]	2000	Biopsies pre hormonal manipulation.	73	0	0	73	IHC	P53 + = 14/73 (19.2%). 6/14 P53+ progress 8/59 p53- progress	P53 positive patients correlated with early relapse.

Table11: The relationship between p_{53} and outcome in patients with extra prostatic disease.

Ref.	Year of pub	Type of specimen	No of cases	No localised	No locally invasive	No with metastasis	Method	P53	Conclusion
[245]	1998	43 patients with hormone refractory disease.				43	IHC	22/43 (51%) p53+ in bone marrow.	Did not correlate with outcome
[238]	1998	Lymph nodes + Rad Prost	56	39	17	56 nodes 19Bone metastasis.	IHC	5/41 Primary tumours p53+ 7/41 lymph nodes p53+	P53+ had no prognostic significance.
[78]	1999	Rad Pros + Lymph nodes	220	0	0	220	IHC	109 Rad Pros p53+ 83 Lymph nodes p53+ The majority of patients then had LHRH.	P53 expression in the primary tumour was concordant with p53 presence in nodes. The presence of p53 expression in the nodes was an adverse prognostic factor for survival.

Table 12: The relationship between p_{53} in pre op biopsies and outcome.

Ref.	Year	Specimen	Number	Method	Stage	Results	Conclusion
[239]	1997	Pre treatment biopsies Mean follow up 35.5 (range 1 –132 months). Treatment with Rad pros (49), hormonal therapy (89), or surveillance (8).	146	IHC	$T_{1-2}N_0M_0$=50, $T_3N_0M_0$=28, $T_{1-4}N_1M_0$=9, $T_{1-4}N_1M_1$=59,	$T_{1-2}N_0M_0$ p53 + = 10/50, $T_3N_0M_0$ p53 + =8/28, $T_{1-4}N_1M_0$ + $T_{1-4}N_1M_1$ p53 + = 22/68. P53+ = 10% staining +	P53 positive correlates with shorter progression free survival. Bcl2 positivity an independent indicator of poor prognosis.
[63]	1998	Pre-tx biopsies. Follow up 5 years, heterogeneous treatment with Rad pros, DXT, hormonal therapy, TURP, or surveillance.	111	IHC	?	16% p53 positive. 20% bcl2 positive. 7.5% Ki67 positive.	P53 + correlated with high-grade tumour. Univariate analysis showed that high Gleason score, high percentage of tumour, high Ki-67 LI, and p53 positive purports high percentage of death. But multivariate high Ki-67 only independent predictor.
[55]	1999	Pre treatment biopsies and radical prostatectomy specimens. Mean follow up38 months (range 6 to 114 months). Treatment with radical prostatectomy.	76	IHC	$T_{1-2}N_0M_0$=37, $T_3N_0M_0$=39,	19/22 with relapse are p53+ on biopsy. 20/40 relapse free are p53+ on biopsy.	Biopsy p53 + in multivariate analysis correlated with poor outcome. Aberrant p53 observed more often in biopsies than in radical prostatectomy specimens.
[364]	1999	Comparison of needle biopsies (129) with rad prostatectomy(199) specimens	129	IHC	?	Rad Pros 132/193 p53+ Needle biopsy 64/129 p53+	Immunostaining of biopsies not predictive of recurrence. Poor correlation between biopsy and rad prostatectomy specimens, but as the editorial suggests the biopsies were done at many institutions and this may be a technical problem.

Table 13: The relationship between aberrant p_{53} and prostatic intra epithelial neoplasia.

Ref.	Year	Specimen	Number	Method	Stage	Results	Conclusion
[175]	1995	Rad Pros	40	IHC	$T_{1-2}N_0M_0$=40	9/40 PIN p53+ 13/40 CaP p53+	Ca P & PIN from 7 common patients demonstrated intense p53 staining.
[426]	1998	Rad Pros (27) TURP (2)	29	PCR-SSCP		3/29 PIN p53 + 5/29 CaP p53 +	2 patients had mutations in both Ca P & PIN, but these were distinct mutations.
[187]	1998	Rad Pros (9 pts with neoadjuvant LHRH)	44	IHC	$T_{1-2}N_0M_0$=26, $T_{3}N_0M_0$=18,	0/44 PIN p53 + 2/43 CaP p53 +	Some pts with LHRH, low incidence of p53.
[377]	2000	Rad Pros (14 pts with neoadjuvant LHRH)	27	PCR-SSCP	$T_{1-2}N_0M_0$=11, $T_{3}N_0M_0$=16,	6/27 PIN had p53 mutations 8/27 Ca had p53 mutations. In only 2 cases did both Ca and PIN have mutations. Both of these mutations were different.	Increase in p53 mutation with stage. Different mutations in p53 in PIN and Ca imply PIN arises from a different clone.

Table 14: The relationship between aberrant p_{53} and metastatic prostate cancer.

Ref.	Year	Specimen	Number	Method	Stage	Results	Conclusion
[387]	1993	Primary tumours	68	IHC Flow Cytometry	?	9/68 p53+	P53 + tumours were higher stage & Gleason score. + progressed more rapidly.
[103]	1994	TURP (20) + Lymph node(15)	35	IHC PCR-SSCP	?	2/20 TURP p53 + 2/15 Lymph node p53+	Total concordance between PCR-SSCP and IHC. P53 mutation is a late and infrequent event.
[29]	1995	50 hormone sensitive + 34 hormone resistant	84	IHC PCR-SSCP	?	6/34 hormone res p53+ 19/50 hormone sens p53+	A significantly higher proportion of tumours with mutations were poorly differentiated compared with tumours without mutation ($P < 0.04$). p53 mutation is a late event in the progression of the disease
[110]	1995	Rad Pros (39). Rad Pros/TURP + mets/nodes (47)	86	IHC PCR-SSCP	$T_{1-2}N_0M_0 = 18$ $T_{3-4}N_0M_0 = 21$ $T_{1-4}N_1M_1 = 47$	0/18 $T_{1-2}N_0M_0$ p53+ 2/21 $T_{3-4}N_0M_0$ p53+ 4/47 $T_{1-4}N_1M_1$ p53+, & 11/47 mets/nodes p53+.	4/47 pts with mets had abnormal both in prostate and lymph nodes. 7/47 had mutation at mets only. P53 mutation associated with advanced stage.
[174]	1995	36 primary cases. 17 positive lymph nodes. 14 bone mets	68	IHC	?	10/36 primary tumours positive 9/36 were Gleason score>7. 10/17lymph nodes positive. 6/14 bone mets positive.	Metastatic tumours express more aberrant p53 than primary tumours.

Table 14 (cont): The relationship between aberrant p_{53} and metastatic prostate cancer.

[259]	1995	Rad Pros (29), frozen section of primary tumour (9), lymph node (12), and bone marrow (1).	51	IHC, PCR-SSCP	?	6/38 primary tumours had aberrant p53. 3/13 mets/nodes had aberrant p53.	Metastatic tumours display higher p53 than primary.
[10]	1996	Biopsy, TURP, Rad Pros. Treatment heterogeneous.	77	IHC	$T_{1-2}N_0M_0$=18, $T_3N_0M_0$ =14, $T_{1-4}N_1M_1$ =45	23/77 + for p53, 14 of the 23p53+ patients were refractory to hormonal manipulation. 37/77 + for bcl2,	23/77 + for p53, 37/77 + for bcl2, P53 staining corresponded to hormone resistance. In 14/21 (66%) of cases (p=. 0012), increased p53 +bcl2 corresponded to hormone resistance in 13/16 (81.2%) of cases (p<. 0001).
[245]	1997	Bone Marrow biopsy in pts treated with LHRH.	43	IHC	$T_{1-4}N_{0-1}M_1$ =43	22/43 P53+	P53 expression did not correlate with tumour burden or outcome. Bcl2 expression was inversely correlated with p53 expression.
[142]	1998	Rad pros + prostate fossa biopsy (18).	18	IHC	$T_{1-2}N_{0-1}M_{0-1}$=11, $T_3N_{0-1}M_{0-1}$ =6, $T_4N_{0-1}M_{0-1}$ =1	Primary p53 + = 1/18 Recurrent p53 + = 7/18	Genotype of recurrence is more aggressive than primary lesion.
[254]	1998	Bone Marrow biopsy in pts treated with LHRH.	17	IHC RT-PCR+SSCP	$T_{1-4}N_{0-1}M_1$ =17	10/17 P53+ staining 8/10 abnormal on RT-PCR+SSCP	71%(12) of the bone marrow samples had aberrant p53; RT-PCR detected 2 missed by IHC.

Table 15: Miscellaneous papers relating p_{53} and prostate cancer.

Ref.	Year	Design of study	Specimen	Number	Method	Stage	Results	Conclusion
[215]	1998	Detect autoantibodies for p53 in sera.	Sera	73	ELISA	$T_{1-4}N_0M_0$=62, $T_{1-4}N_0M_1$=11,	2/62, 0/11 + for antibodies.	2 patients with localised disease had antibodies; none of the patients with metastatic disease had antibodies. Both of these patients died in 2-6 months.
[240]	1998	Relationship between p53 and p21.	Archival specimens	60	IHC	$T_{1-2}N_0M_0$=16, $T_{3-4}N_0M_0$=10, $T_{1-4}N_{0-1}M_1$ =34,	$T_{1-4}N_0M_0$=10/26 p53 +, $T_{1-4}N_{0-1}M_1$ =23/34 p53+	P21waf1 downstream messenger of wtype p53 not activated by mutant p53, p53+/p21-poor prognosis, compared to p53+/p21+
[201]	1999	Hormone refractory tumours (30) versus matched untreated primary (24).	Archival specimens	30	FISH IHC	$T_{1-2}N_0M_0$=6, $T_{3-4}N_0M_0$=24, $T_{1-4}N_{0-1}M_1$ =10,	Primary tumours = 4/24 p53+, Hormone refractory = 12/30 p53+	P53 positive tumours show androgen receptor gene amplification, which may allow these tumours to grow in a low androgen milieu.
[193]	2000	Relationship between p53 and 15-lipoxygenase in CaP	Fresh frozen	48	IHC	?	36/48 15 lipoxygenase +. 19/48 p53 +	Correlation between p53, 15-lipoxygenase, and Gleason score. Mutant p53 upregulates 15 lipoxygenase expression.
[375]	2000	Relationship between p53 and VEGF IN CaP	Archival specimens	55	IHC	$T_{1-2}N_0M_0$=31, $T_{3-4}N_0M_0$=24,	12 overexpression of P53	No correlation between p53 and VEGF

3.4 BCL_2

The Bcl_2 family of related proteins constitute important regulators in the control of apoptosis (programmed cell death) [7]. There are over a dozen members of the bcl_2 family some suppress and some promote apoptosis. The bcl_2 gene is highly conserved. The bcl_2 gene was originally detected in a proportion of B-cell follicular lymphomas at the t (14:18) chromosomal translocation breakpoint [202]. The bcl_2 proto-oncogene is located on chromosome 18q21 and codes for a 26 kDa, 239 amino-acid protein with a hydrophobic carboxy terminal. Bcl_2 protein has been located to the mitochondrial membrane [163], nuclear envelope, and the endoplasmic reticulum[388]. Bcl_2 may inhibit apoptosis by regulating the mitochondria permeability and the release of pro apoptotic enzymes. The pro apoptotic members of this family are cytosolic, following the initiation of apoptosis the pro apoptotic members undergo a conformational change and integrate into membranes. An additional characteristic of this family is their frequent ability to form homo as well as heterodimers, suggesting neutralising competition between these proteins.

In IL-3 dependent myeloid and lymphoid cell lines, bcl_2 inhibited apoptosis induced by IL-3 withdrawal without stimulating proliferation [401]. Implying therefore, that the malignant potential of bcl_2 is mediated by its ability to promote cell survival rather than proliferation. In benign prostatic tissue, the

basal cells express bcl_2 [163].

In prostate cancer, hormone manipulation achieves its effect through activation of apoptosis. Bcl_2 is an inhibitor of apoptosis induced by a number of mechanisms – growth factor withdrawal, glucocorticoid withdrawal, heat shock, and chemotherapeutic agents.

The initial evidence demonstrating that bcl_2 imparts a growth advantage following androgen ablation was obtained using the hormone sensitive LNCaP cell lines, the cell lines were transfected with bcl_2 and androgen withdrawn from the growth medium. The native cell lines did not grow in the androgen free medium; where as the rate of growth of the transfected cell lines was not affected by androgen withdrawal [313]. This model was further developed in xenografts of these cell lines. The cells were injected into nude mice. Following castration, the bcl_2 expressing tumours exhibited a significant growth advantage. Therefore, bcl_2 enables prostate cancer cells to remain viable despite androgen ablation [22].

The relationship between androgen ablation and bcl_2 expression was studied in castrated animals at intervals of 1 to 10 days post castration. Messenger RNA for bcl_2 was extracted from the ventral lobe of the prostate. This study demonstrated that castration lead to an increase in the levels of mRNA for

bcl_2. The increase was greatest at ten days. This effect could be negated with subcutaneous injections of testosterone[246]. Therefore androgen ablation itself may lead to the selection of an androgen insensitive phenotype [246].

It has been suggested, using gamma irradiated LNCaP cells that bcl_2 protein may inhibit the nuclear import of p_{53} protein and so prevent apoptosis, the exact mechanism by which bcl_2 inhibits the transfer of cytoplasmic p_{53} protein into the nucleus is unclear[21]. Structural studies of the bcl_2 protein demonstrate similarities to the pore-forming domains of bacterial toxins, which suggest a role in modulation of membrane function [83]. This evidence suggests a gate keeper role for bcl_2.

A set point exists in most cells for the ratio of bcl_2 family dimmers, e.g. bcl_2: bax and this determines the sensitivity of a cell towards survival and apoptosis [292]. Internal and external messengers can further regulate this ratio [335]. Bax may be negatively selected during progression of cancer implying that it may function in a tumour suppresser role independent of p_{53} [317].

Phosphorylation of bcl_2 at specific serine residues can lead to its inactivation and so can inhibit its anti-apoptotic functions [150]. P_{53} can mediate bcl_2 phosphoryaltion and apoptosis via activation of G proteins Cdc42. Cdc42 is a

GTPase, and p_{53} promotes apoptosis through activation of this G protein [386]. Phosphorylation of the bcl_2 protein also leads to decreased binding to bax.

The anti-cancer drug taxol induces bcl_2 phosphorylation and subsequent apoptosis of prostate cancer cell lines[149]. Taxol also has an effect upon microtubule, it inhibits microtubule depolymerisation during the cell cycle[329], Haldar et al have used this evidence to suggest that microtubule damage may lead to cell death through a mechanism that involves bcl_2 phosphorylation [148]. Taxol therefore induces bcl_2 phosphorylation and cell death, whereas drugs, which damage DNA, do not induce bcl_2 phosphorylation but induce apoptosis through a p_{53} dependant pathway. This has led Haldar et al to suggest that bcl_2 is the guardian of microtubule integrity [148]. Microtubules are important in chromosome segregation and alteration could lead to genomic instability and cell death.

Antisence oligodeoxynucleotides (ODN) are chemically modified stretches of single stranded DNA that are complimentary to mRNA regions of a target gene. ODNs inhibit gene expression by forming RNA/DNA duplexes. Using antisence bcl_2 ODNs and the agent paclitaxel in LNCaP cell lines implanted into nude mice, Leung et al demonstrated that serum PSA levels mirrored tumour volume. Mean serum PSA levels returned to or were above precastration levels by 11 weeks post castration in mice treated with bcl_2

ODNs or paclitaxel alone, but remained 90% below pre-castration levels in mice treated with combined antisense ODNs and paclitaxel. Therefore the combination of antisense bcl_2 ODN and paclitaxel led to a decrease in tumour volume and delay in androgen independent progression [222]. Similar results were obtained by Miyake et al except they also demonstrated using Shinogi tumour cells that antisence bcl_2 ODNs could be tumour specific and there was no reduction in bcl_2 expression in normal tissue [132;255;256].

Transgenic mice have been used to study the pathogenesis of prostate cancer. The most extensively used model is the TRAMP (transgenic adenocarcinoma mouse prostate) model. In this model probasin directed expression of SV40 early genes in the prostate results in progression from hyperplasia to metastatic disease by 28 weeks of age. This model was used to target bcl_2 to the prostate and generate the BxT (bcl_2 x TRAMP) mouse. The BxT mice displayed increased prostate size associated with decreased apoptosis, earlier development of PIN and invasive carcinoma. By 20 weeks 100% of the BxT mice exhibited evidence of prostate cancer compared to only 50% of the TRAMP mice. The rate and incidence of tumour metastases was the similar for the TRAMP and BxT mice. The transgenic bcl_2 did not result in malignant transformation of the prostate gland [58].

Transfection of bcl_2 into LNCap and PC-3 cell lines followed by exposure to

ionising radiation led to the bcl_2 transfected cells having decreased apoptosis although both the controls and the transfected cells did not form colonies after exposure to radiation [211]. This elevation in bcl_2 resulted in a significant suppression of radiation induced apoptosis at 48 hrs, but after longer periods post irradiation even the bcl_2 expressing cells underwent apoptosis. This suggests that bcl_2 over expression does not provide a permanent protection against radiation induced apoptosis but rather it delays the onset of the process [211].

The angiogenic switch stimulates angiogenesis at a distinct time during tumorigenesis. Through the stimulation of angiogenesis and the inhibition of apoptosis tumour cells attempt to negate the effect of hypoxia. This was investigated using prostate cancer cell lines transfected with bcl_2. These cell lines were subjected to hypoxic conditions in vitro. Cells with transfected bcl_2 expressed significantly more vascular endothelial growth factor (VEGF) than the controls [116]. The cells were then injected subcutaneously into nude mice; the bcl-transfected tumours grew faster, expressed more VEGF and a greater micro vessel density [116]. The bcl_2 transfected cells were implanted in mouse corneas, and six of the eight corneas implanted with transfected cells had evidence of neovascularisation compared to none of the controls. The authors of his study concluded that increased VEGF expression and the resulting increased vascularisation associated with this are essential components of the

in vivo growth advantage conferred to tumours expressing bcl_2 [116].

Bcl_2 over expression occurs in colorectal adenomas, cervical intra-epithelial neoplasia and other pre-malignant lesions. The basal cells of the prostatic epithelium probably house the prostatic stem cell population [40]. These cells are resistant to androgen dependant apoptosis, where as secretary luminal cells require androgens and undergo apoptosis when deprived of androgens. The expression of bcl_2 is not uniform within the prostate, the transitional and peripheral zones exhibit strong expression for bcl_2 in the basal cells but not in the secretary luminal cells. The central secretory zone does express heterogeneous expression of bcl_2[41]. In a series of 216 foci of high grade PIN from 25 radical prostatectomy specimens, Bcl_2 expression was restricted to the basal cells in 169 specimens (78%), and was present in secretory luminal cells in 22%. No difference was detected in bcl_2 expression with and without adjacent cancer. There was a marked inverse relationship between the expression of the androgen receptor and bcl_2. In the transition and the peripheral zones the secretory luminal epithelium strongly expressed the nuclear androgen receptor. The basal cells were unreactive for androgen receptor staining. High grade PIN expressing bcl_2 in the basal cells only stained for the androgen receptor in the luminal secretory cells. Where as high grade PIN over expressing bcl_2 in the luminal cells showed a marked reduction in expression of the androgen receptor. The inverse correlation between bcl_2

and androgen receptor expression was highly significant. It has therefore been postulated that the aberrant expression of bcl_2 in sub-sets of PIN may indicate resistance to androgen dependent cell death, and probably reflect reduced androgen-responsiveness of the dysplastic epithelium [41].

The development of androgen resistance can be associated with bcl_2 expression, it can also occur independently of bcl_2. This has been demonstrated by work carried out on the Dunning AT-3 cell line that is highly malignant but does not express bcl_2 [122].

3.41 Bcl_2 and Prostate Cancer

A literature review was carried out using the Medline database. The initial keyword used was bcl_2 and the second was prostate neoplasm. For the first keyword a total of 6302 references were detected and for the second 33298. Theses two searches were combined and the number of references decreased to 160 references. The abstracts of these 160 citations were reviewed and a total of 50 pertinent references were reviewed in detail [10;15;16;20;21;21;22;27;41;43;55;58;60;66;74;76;116;122;125;132;142;148;173;179;185;187;191;204;211;217;222;227;235;238;239;245;246;255;256;274;308;315;341;348;364;372;381;382;385;385;394].

3.42 Bcl_2 and Radical Prostatectomy.

To study the significance of bcl_2 a cohort of 137 consecutive radical prostatectomy specimens obtained from patients who underwent radical prostatectomy between 1978 and 1993 at the University of Bassel were examined for over expression of bcl_2 and p_{53} [60]. 40 patients were stage pT_2, 93 patients were pT3, no stage was given for 4 patients, 103 patients were pN0 and 34 were pN1/2. Follow up data was available from 115 patients with a mean follow up of 5.2 years. Only eight patients had died, therefore survival data was not discriminatory, but progression data was available. 54 patients had received adjuvant local radiotherapy. Orchiectomy was performed upon six patients at the time of prostatectomy. Progression was documented in 37 patients based upon PSA increase in 29 patients, biopsy in 3 and a positive bone scan in 5. Bcl_2 over expression was associated with higher stage, shortened disease free survival. This study also stained for Ki-67 and p_{53} expression. Progression analysis showed that patients with bcl2 positive and Ki 67 positive tumours had the shortest progression free survival, and those with bcl_2 and Ki 67 negative tumours had the longest disease free survival[60].

The group from the Walter Reed Army Medical Centre, Washington carried out immunohistochemistry on 175 radical prostatectomy specimens for both p_{53} and bcl_2 [20]. One hundred and seventy four of the patients had capsule confined disease. Over expression of bcl_2 was detected in 47 of 175 (26.9%)

patients, these patients had a statistically significant higher 5 year failure rate when compared to bcl_2 negative patients i.e. 67.0% versus 30.7%. Combining the bcl_2 positive and the p_{53} positive patients, the failure rate was 75.3%, compared to a failure rate of only 20.4% if both bcl_2 and p_{53} were negative[20]. Similar results were obtained using a consecutive series of 208 radical prostatectomies and a mean follow up of 4 years. Twenty-eight cases (13%) expressed bcl_2; bcl_2 positivity was an independent prognostic marker in a multivariate analysis[194].

Specimens obtained from a heterogeneous series of 40 patients with prostate cancer were used to determine the association between disease progression and bcl_2, waf-1, and p_{53}. Twelve patients over expressed bcl_2, of these ten progressed; twenty-eight patients did not express bcl_2 of these 19 progressed. There was no correlation between the expression of bcl_2, clinical grade, stage and progression [66].

Apakhama and colleagues studied seventy seven patients with prostate cancer at various stages were followed up for a median of 30 months and the importance of p_{53} and bcl_2 assessed. The age range was from 46 to 88 years. Forty-five patients (58.4%) had metastases, 37 patients stained positively for bcl_2. Seventeen of these patients stained positively for both p_{53} and bcl_2, of these 16 had had hormonal manipulation, and 13 had hormone refractory

disease. These workers postulated that the combined detection of p_{53} and bcl_2 may be useful in detection of hormone refractory disease [10].

A study of 235 patients, 172 with no metastases which had been treated by a number of modalities including seven with radical prostatectomy, 4 with radical radiotherapy, 22 with oestrogen, 61 with orchiectomy, 33 patients had a TURP and 82 patients were treated conservatively [227]. 71% of the tumours were bcl_2 negative, 18% were weakly positive, and 11% were strongly positive. The expression of bcl_2 correlated with higher tumour stage, metastatic disease, and high grade and in a univariate analysis with poor survival, but in a multivariate analysis the expression of bcl_2 had no prognostic significance. In a study of 41 patients with lymph node metastases from a Japanese centre, bcl_2 expression was observed in both primary tumours and metastases from 14 tumours [238]. Bcl_2 expression was found to be of no prognostic value.

3.43 Bcl_2 and Radical Radiotherapy.

A study of 43 patients who had been treated with definitive radiotherapy for prostate cancer with a follow up period of at least 3 years showed increased bcl_2 staining predicted relapse[173]. A study of 54 patients, who were treated with radiotherapy, showed that increased staining of bcl_2 or p_{53} predicted relapse [341].

3.44 Bcl_2 and Watchful Waiting.

In the Scandinavian nations prostate cancer is often treated conservatively, a study of 221 men diagnosed by TURP chips and treated conservatively with a median follow-up of 15 years demonstrated that 114 cases over expressed bcl_2. This correlated with clinical stage and disease specific survival. If the tumours were further divided into those that were p_{53} positive and bcl_2 negative compared to p_{53} positive and bcl_2 positive, those in the first group did better than the second. But dividing the p_{53} negative group into those that were bcl_2 positive versus those that were bcl_2 negative made no difference to disease free survival [43].

3.45 Bcl_2 and Recurrence.

The relationship between the primary tumour and localised recurrence after definitive treatment was studied in a series of recurrent tumours after definitive surgery (n=18) or radiotherapy (n=13). There was an increase in the expression of bcl_2 in recurrent tumours when compared to the primary lesion but this did not reach significance [142]. This was further studied in a series of 32 patients who had failed radiotherapy and had pre and post radiotherapy specimens, prior to radiotherapy 14 patients expressed bcl_2 and post radiotherapy 19 expressed bcl_2. In the group of patients that had no disease

recurrence post radiotherapy only one out of the 12 patients expressed bcl_2. This was highly significant. If both p_{53} and bcl_2 were examined together significantly more patients had p_{53} and bcl_2 immunopositivity following radiation failure, 48%, compared to 8% in those that were successful [173].

3.46 Bcl_2 and Prostate biopsies.

The role of prostate biopsies at predicting outcome was studied in 146 patients. Bcl_2 positivity was detected in 29 patients. Bcl_2 positvity was detected in 23.7% (18/76) of patients with localised disease, and in 15.7% (11 of 70) of patients with metastatic disease. Using multivariate analysis bcl_2 positivity was an independent predictor of outcome [239].

The group from Bristol compared preoperative biopsy assessment of bcl_2, p_{53}, CD-44 and e-cadherin with the expression of these markers in the respective radical prostatectomy specimen [55]. Radical prostatectomy and biopsy specimens were examined from a total of 76 patients; aberrant staining for bcl_2 was detected in eight preoperative biopsies and in 15 radical prostatectomy specimens. Brewster et al did not comment if preoperative biopsy staining for bcl_2 correlated with expression of bcl_2 in the radical prostatectomy specimens. Preoperative bcl_2 was not a significant predictor of outcome. Using Cox regression no direct relationship between bcl_2 over expression and p_{53} protein accumulation could be established. Using a multivariate analysis post-

operative p_{53} and bcl_2 were independent prognostic predictors of disease free survival [55].

Similarly, in a series of 117 preoperative samples from radical prostatectomies carried out at the Walter Reed Medical Centre the preoperative biopsies did not correlate with out come [364]. Forty-nine patients had evidence of disease recurrence, and 18 preoperative samples had evidence of bcl_2 over expression. Comparison between biopsy and prostatectomy staining revealed no concordance. Biopsy samples could not predict outcome [364].

Biopsies have also been used to predict outcome in-patients treated with definitive radiotherapy. In a series of 52 patients with pre radiotherapy biopsy material available for immunostaining, thirteen stained positively for bcl_2. Of these, 11 patients (84.6%) failed radiotherapy, in the bcl_2 negative group only 16 of the 39 patients (41.1%) failed radiotherapy. This result was significant [341].

3.47 Androgen deprivation and Bcl_2

The role of androgen ablation in changing the expression of bcl_2 and p_{53} was studied using radical prostatectomy specimens obtained from 106 patients was compared with 71 pre treatment prostate biopsies[74]. The patients prior to surgery had 3 months of LHRH treatment. Eleven of the 106 radical

prostatectomy specimens over expressed bcl_2 and nine of the 71 pre treatment biopsies expressed bcl_2. No correlation was found between the expression of bcl_2 in the biopsies and prostatectomies, four cases were positive in both samples, four only in the biopsies and three only in the prostates. No correlation existed with bcl_2 over expression and clinical outcome, preoperative PSA, or accumulation of aberrant p_{53} [74]. However the data from this study must be viewed with caution since in studies comparing biopsies and radical prostate specimens, the expression of bcl_2 in material obtained from biopsies does not correlate with the expression of bcl_2 obtained from radical prostatectomy samples.

An earlier study comparing two groups of radical prostatectomies, one that had neoadjuvant hormonal therapy (n=28) with ones that had not (n=51), found increased expression of bcl_2 after neoadjuvant hormonal ablation[394].

A study of 69 heterogeneous patients with prostate cancer demonstrated a trend towards increased bcl_2 expression post androgen deprivation [16]. However in this study the authors could not demonstrate any correlation between the expression of bcl_2 and apoptosis, p_{53}, or clinical outcome.

This effect of increased bcl_2 with castration was further investigated in series 15 responders and 13 non-responders of androgen ablation[372]. The patients

had biopsies pre androgen ablation and one week after treatment. Both groups had an increase in bcl_2 expression associated with androgen ablation, but the responders had significantly increased bcl_2 expression as compared to the non-responders [372].

3.48 Bcl_2 and High Grade Prostatic Intra Epithelial Neoplasia (PIN).

High-grade prostatic intra epithelial (PIN) neoplasia is a putative precursor of prostate cancer, and the incidence of bcl_2 expression was studied in PIN using 43 radical prostatectomy specimens. Bcl_2 was over expressed in 1 of the 43 cancers (2.3%), and in 15 of the 43 PIN specimens (34.9%) [187]. These authors suggested that the foci of bcl_2 positive PIN might not be the direct precursors of invasive cancer.

3.49 Bcl_2 and Metastases.

Prostate cancer often metastasises to the bone marrow. The accumulation of bcl_2 was studied in 43 bone marrow samples obtained from patients with bone metastases. All patients had been treated with at least hormonal ablation. Bcl_2 was detected in 14 of the 43 cases. Six of these cases exhibited only positive staining for bcl_2, and eight expressed both p_{53} and bcl_2. The expression of bcl_2 protein did not significantly influence patient survival [245].

The relationship between bcl_2 expression and metastasis was in hormone naïve

patients showed that 5 of 30 (17%) patients with lymph node disease had positive bcl_2 staining and 14 of 27 (52%) patients with bone metastasis had positive staining in bone marrow samples [122]. Therefore bcl_2 expression increases with increasing stage.

3.50 Bcl_2 and Bax.

Prostate cancer is often managed with external beam radiotherapy. The prognostic importance of bcl_2 and bax were investigated in a series of 41 patients who had undergone external beam radiotherapy for the management of localised carcinoma of the prostate. 21 patients responded to radiotherapy and 20 failed to respond as determined by a PSA nadir of 1.0ng/ml. Bcl_2 expression was significantly higher in the non-responders and bax expression was higher in the responders. The ratio of bcl_2/bax was significantly higher in the non-responder when compared to the responders' [235].

Table16: The relationship between bcl_2 expression and outcome in patients with radical prostatectomy.

Ref.	Year of pub	Type of specimen	No of cases	No localised	No locally invasive	No mets	Method	Results/Conclusions
[60]	1996	Radical Prostatectomy	137	40	93	34	IHC	T2 2/40 (5%) + T3 29/93 (31%)+ N0 25/104 (24%)+ N1/2 8/34 (24%)+ Bcl-2 expression increased with stage.
[20]	1996	Radical Prostatectomy	175	T1=25 T2=149	T3=1	0	IHC	47/175 (27%) Bcl2 positive. Correlated with poor survival
[10]	1996	Heterogeneous	77			45	IHC	37(48%) positive for bcl2. 20/37 androgen resistant
[66]	1997	Heterogeneous	40	T1=10 T2=5	T3=11 T4=13	21	IHC	12/40 (30%) Bcl-2 + 10/12 progressed. No correlation with bcl-2 + and clinical outcome
[227]	1997	Heterogeneous	235	T1= 94 T2 = 56	T3=96 T4=19	63	IHC	18% weakly positive 11% strongly positive. Bcl-2 correlated with poor survival, but of no an independent prognostic value.
[194]	1998	Radical Prostatectomy mean follow up 4yrs.	208	111	97	8	IHC	28/208 (13%) + Bcl-2 correlated in a multivariate analysis with poor outcome.
[238]	1998	Biopsies and Radical Prostatectomies	41	?	?	41	IHC	14/41 (34%) bcl-2 +. No correlation with bcl-2 + and clinical outcome.
[142]	1998	Radical Prostatectomy	18	11	7	5	IHC	Increased expression of bcl-2 in recurrence. Not significant.

[142]	1998	Biopsies DXT	13	13	0	0	IHC	Increased expression of bcl-2 in recurrence. Not significant.
[74]	2000	Radical Prostatectomy	106	58	31	17	IHC	11/106 + No correlation with bcl-2 + and clinical outcome
[421]	2003	Radical Prostatectomy	70	70			IHC	3/70 + Increased expression of bcl2, predicted relapse.
[319]	2005	Radical Prostatectomy	131	60	71	10	IC	13/131 bcl2+ Increased expression of bcl2, predicted relapse.

Table17: The relationship between bcl_2 expression and outcome in patients treated with radical radiotherapy.

Ref.	Year of pub	Type of specimen	No of cases	No localised	No locally invasive	No mets	Method	Results/Conclusions
[235]	1998	Pre DXT biopsies	41	41	0	0	IHC	Significantly higher bcl-2/bax ratio in radiation failures.
[173]	1998	Biopsies and rad prostatectomy	43	43	0	0	IHC	1/12 bcl-2 + in the radiation success, 14/31 bcl-2 + in the radiation failures. Bcl-2 was found in significantly more radiation failures.
[341]	1999	Pre-DXT biopsies	52	52	0	0	IHC	13/52 (25%) bcl-2 +. 11/13 bcl+ cases failed treatment with DXT.
[328]	2003	Needle biopsy	40	29	11	0	IHC	11/20 patients with recurrence overexpressed bcl2, 0/20 in the recurrence free group.

Table18: The relationship between bcl_2 expression in pre operative biopsies and outcome.

Ref.	Year of pub	Type of specimen	No of cases	No localised	No locally invasive	No mets	Method	Results/Conclusions
[239]	1997	Biopsies	146	50	28	68	IHC	29/146 bcl-2 +. In multivariate analysis an independent prognostic indicator.
[372]	1998	Pre and post castration biopsies in 15 responding patients and 13 patients not responding to hormonal therapy.	28	7	21	20	IHC	Bcl-2 expression increased significantly with androgen ablation, significantly more so in the responders.
[364]	1999	Pre op biopsies	117				IHC	18/117 (15%) bcl-2 +. No correlation with bcl-2 + and clinical outcome.
[55]	1999	Pre op biopsies	65				IHC	8/65 bcl-2 (12%) + Pre-op Bcl-2 did not correlate with outcome.
[74]	2000	Pre op biopsies	71					9/71 bcl-2 + No correlation with bcl-2 + and clinical outcome
[43]	2000	TURP biopsies	221	125	96	60	IHC	Patients treated with watchful waiting. 114 (52%) cases + for bcl-2. Correlated with clinical stage and disease specific survival.

Table19: The relationship between bcl_2 expression and metastasis.

Ref.	Year of pub	Type of specimen	No of cases	No localised	No locally invasive	No mets	Method	Results/Conclusions
[122]	1996	Lymph node (30) & Bone marrow(27)	57	0	0	57	IHC	5/30 lymph node bcl-2 + 14/27 bone marrow bcl-2 + Bcl-2 expression increases with stage.
[245]	1997	Bone marrow.	43	0	0	43	IHC	14/43 (32%) bcl-2 +. No correlation with bcl-2 + and clinical outcome.

3.60 BAX

Bcl_2 associated X protein, or bax is a proapoptotic molecule, which dimerises with the apoptotic Bcl_2. This dimerisation inhibits the action of bax. The dimerisation takes place at the homology regions and all family members share at least one of the four regions- BH1, BH_2, BH3 and BH4[424]. The truncated form of Bax, expressing only BH3, is sufficient to induce apoptosis and antagonise the effects of ant-apoptotic members of the family [156]. Induction of bax is promoted by the initiation of apoptosis. This causes the monomeric bax to translocate from the cytosol to the mitochondrial membrane; here it becomes an integral part of the membrane and cross link's as a homodimer, resulting in cell death by the release of cytochrome c from the mitochondria[141]. This ability to target the mitochondrial membrane is regulated by the terminal 20 amino acids. Bcl_2 inhibits bax-mediated apoptosis. The bax gene promoter is p_{53} responsive and so p_{53}-binding results in transactivation of the bax gene. Therefore, bax up regulation is one method by which p_{53} exerts its pro-apoptotic action [261].

A literature review was carried out using the Medline database. The initial keyword was prostate cancer and this revealed 35234 citations, the second keyword was bax and the citation count was 23802. When the two citations were combined the number decreased to 194. The abstracts were reviewed

and a total of 15 citations were selected as pertinent 9;81;101;117;122;132;187;199;204;226;230;235;235;313;330;422.

3.61 Incidence of Bax

The incidence of bax was studied in 64 radical prostatectomy specimens. Bax was detected in every specimen [204]. In Asians the bax was present in 47 of 62 radical prostatectomy specimens i.e. 76%. In a univariate analysis bax expression predicted a significantly poorer survival[81].

3.62 Bax and radiotherapy for prostate cancer.

A retrospective review of 41 patients who underwent radiotherapy with curative intent showed that the patients could be divided into two groups depending upon the PSA response and the presence of tumour in the post operative biopsies. This demonstrated no difference in bax immunoreactivity between responders and nonresponders[235], but the staining of bcl_2 was increased in non responders and so the ratio of bcl_2/bax was higher in non-responders[235]. The importance of the bax: bcl_2 ratio was further assessed by using synthetic peptide sequences from the BH3 domain of bax, this resulted in binding between the BH3 peptides and bcl_2 and so blocking bcl_2/bax heterodimerisation. The excess of released bax led to an increase in apoptosis[117].

Using adenovirus vectors, bax was over expressed in four different cell lines. In all the cell lines bax over expression caused cytochrome c release and initiation of the capsase mediated apoptosis pathway. Bax over expression by passed the anti apoptotic effect of bcl_2, and caused apoptosis in cell lines resistant to chemically induced apoptosis [226]. This finding has been confirmed by a second group [230]. Androgen withdrawal is known to cause apoptosis; the presence of androgens in androgen responsive cell lines causes attenuation in the expression of bax [199]. Using an adeno virus construct, which contained the bax gene, linked to androgen responsive elements and with probasin as a promoter. Probasin is a prostate specific promoter, so that the molecule can be over expressed in prostate only. This model has shown that bax expression and so apoptosis could be made to occur in androgen dependant manner, both in vitro and in mice[9].

3.63 Bax and high grade prostatic intraepithelial neoplasia.

In a heterogeneous group of 44 radical prostatectomy specimens, Bax expression can de detected in all cases of PIN[186].

3.70 Ki 67

The name of the Ki-67 protein is derived from its city of origin – Kiel, and the number of its original clone in a 96 well plate. The Ki-67 antigen is a nuclear protein that is present in proliferating human cell [127]. The genetic locus has been assigned to chromosome 10 and consists of 30,000 base pairs[127;345]. Two isoforms of the protein have detected with masses of 320 and 359kD; both contain a large central region with 16 repetitive elements [345]. Homology searches have failed to detect regions of similarity between Ki-67 and other proteins. Although the functional role of Ki-67 is unknown, it is expressed in all stages of the cell cycle (G1, S, G_2, and mitosis) except for G0. Ki-67 immunoreactivity appears to be a valid method to measure proliferation [346]. Ki-67 is a part of the nuclear matrix and of the chromosome scaffold. Initially the use of Ki-67 was limited to frozen section material, however the development of antigen retrieval systems has led to newly developed antibodies being applied to formalin fixed material [196].

The proliferation rate is low in prostate cancer but it may be important in determining tumour progression. Proliferation assessed by mitotic rate [402], S-phase fraction [392;404] and Ki-67 [159;247] has been related to outcome[370]. However at present the clinical significance is far from established. Using archival material and correlating this with outcome it has been shown that proliferation as assessed by Ki-67 immunoreactivity is higher in prostate

cancer than in benign tissue [1:84:389]. Indeed the grade of the tumour is related to the expression of Ki-67[155:260:285]. Prostate hyperplasia and well-differentiated tumours are negative for the expression of Ki-67[285]. The Ki-67 expression can be used as a marker for predicting outcome in prostate cancer[1:155:370]. In prostate biopsies the expression of Ki-67 can predict local extra capsular spread but not metastasis [229].

The cellular proliferation index is also a predictor of recurrence after definite local therapy i.e. radiotherapy or radical prostatectomy [142]. In patients with lymph node metastasis the Ki-67 labelling index of the primary tumour and the lymph nodes had an independent prognostic value where as p_{53} and bcl_2 had no prognostic value[142]. Others have not found Ki-67 so useful [397].

3.71 Why Study Cell Proliferation?

A major stimulus to the study of cell proliferation is the belief that quantification of this fundamental biological process would provide objective data with which to more effectively categorise tumours, predict prognosis and consequently result in tailoring therapy to individual patients. In the past a number of techniques have been used [152:154]. For example, mitotic index estimation by light microscopic examination, in vivo (or in vitro) incorporation of thymidine analogues into DNA during S phase, flow cytometric assessment of DNA index. These methods have significant

disadvantages such as ethical, methodological problems and loss of tissue architecture. An alternative method which is simple, reliable, reproducible and allows retention of normal histology has been developed using antibodies that recognise, by immunohistochemistry, molecules whose expression is linked to the cell cycle [152:244].

3.72 Cell Cycle

The cell cycle describes a co-ordinated series of biochemical alterations that allow exact duplication of the DNA content in a cell (S phase) followed by precise segregation of the DNA into two sets and their subsequent partition into two daughter cells during mitosis (M phase). After mitosis the daughter cells enter a quiescent period of varying duration before commitment to a further cell cycle. The period between one M phase and the next S phase is called Gl. During G1 phase there is growth and preperation of the chromosomes for replication. The period between S phase and the following M phase is called G_2. The cell undergoes preparation for replication during the G_2 phase. Regulated alteration in expression and function of regulatory and structural proteins occurs during the cell cycle. This cyclical alteration in proteins permits the use of antibodies to define antigens expressed in a cell cycle related manner.

Potential antigenic targets for the definition of cells within the cell cycle are

numerous. One of the most widely used antibody is Ki-67 [127], which reacts with a nuclear non-histone protein of 395 and 345 kD present in all active parts of the cell cycle, i.e., GI, S, G_2, and mitosis, but is absent in GO [127;208].

The evidence for the link between the expression of the Ki-67 antigen and the cell cycle is overwhelming [57;337]. The molecule begins to be expressed in mid GI increasing in level through S and G_2 and peaking in M [127]. It is very rapidly catabolised at the end of M. There is good relationship between detectable expression of Ki-67 and growth fraction in several model systems [244;346]. Furthermore, there appears to be no expression of Ki-67 during DNA repair processes unlike some other cell cycle associated molecules (for example PCNA). The function of Ki-67 remains unclear but it may represent a structural protein that maintains the higher order structure of DNA during the important events of mitosis [337]. The major drawback of this antibody is that its application is restricted to fresh or frozen tissues and the epitope does not survive routine histophathological fixation in formaldehyde.

3.73 MIB-1

Recently monoclonal antibodies, MIB 1-3 (MIB for Molecular Immunology Borstel), against recombinant parts of the Ki-67 antigen have been developed. These antibodies are true Ki-67 equivalents, as demonstrated by inimunostaining of fresh specimens, biochemistry, and molecular biological

techniques [73:195]. Formerly immuno-histochemistry on formalin-fixed, paraffin-embedded sections failed to stain for Ki-67. However, when dewaxed microwave oven-processed paraffin sections of formalin-fixed tissues were used, MIB 1 and MIB 3 gave strong nuclear staining of proliferating cells under a variety of normal and neoplastic conditions [73:286]. This method is reproducible, easy to perform at low cost; no additional technical skill is needed and allows a much better recognition of cellular details and, therefore, a better identification of positive cellular subsets. This new method has been successfully applied to tissue sections stored for a long time. The assessment of cell kinetics through the detection of Ki-67 antigen is now possible on archival material.

By immunostaining tissue with a suitable antibody a proliferation index (P.I.) can be calculated as the ratio of nuclear area number positively stained to total nuclear area number in a fixed high power field.

3.80 Ki-67 and Prostate Cancer.

A literature review was carried out using the Medline database. The initial keyword used was prostate cancer and the second was Ki-67. For the first keyword a total of 4250 references were detected and for the second 37565. Theses two searches were combined and the number of references decreased to 145 references. This was further limited to English language abstracts and five

further abstracts were eliminated. The abstracts of these 140 citations were reviewed and a total of 43 pertinent references were reviewed in detail 1:15:30:43:59:62:63:84:90:109:115:142:155:158-160:187:194:197:228:229:238:241:260:274:275:285:286:295:334:338:345:368:379:379:389:393:394:397:398:403:411:414.

3.81 Ki-67 expression and outcome in patients with radical prostatectomy.

In a series of 180 radical prostatectomies with a follow up of 1 to 9 years, MIB1 scoring was high in 168 specimens and absent or low in 12 specimens. The five-year recurrence free survival correlated with MIB1 expression. The authors concluded that MIB 1 expression could be used to determine a subset of patients who are more likely to relapse [30].

In a series of 92 radical prostatectomy patients carried out in the Netherlands, the expression of Ki-67 (MIB 1) correlated with recurrence but this did not reach significance in a multivariate analysis [403].

In a series of 137 consecutive radical prostatectomies, with a mean follow up of 5.8 years available for 108 patients, a Ki-67 labelling index above 7.5% was predictive of recurrence in a multivariate analysis[207]. A similar study of 29 patients using a cut off of 10% also showed a statistically significant association between the proportion of Ki-67 cells labelled and biochemical

relapse[15]. A study of 104 patients focussing on the Ki-67 staining in the area of highest tumour grade with a median follow up of 56 months showed a median staining of 6.7% (range 1.2 – 42.6). Using a cut off of 6.7%, the Ki-67 staining was a predictor of relapse in a multivariate analysis[155].

A recent study of 122 patients who underwent radical prostatectomy selected 22 patients with "metastatic disease" (pT3b-4aN0M0 and pTanyN1M0) and 18 "nonmetastatic" (<=pT3aN0M0). At a mean follow up of 33 (4 – 78) months, the proliferation index predicted relapse in both groups[376].

A study of 20 radical prostatectomy specimens showed that the Ki-67 score is greater for peripheral zone tumours when compared to transition zone cancer[115]; this is in concordance with more aggressive behaviour of peripheral zone tumours.

3.82 Ki-67 expression and outcome in patients with radical radiotherapy.

Animal studies using radiated heterotransplanted DU-145 cell lines and comparing the expression of Ki-67 in the radiated and control groups have not shown a significant decrease in proliferation activity [285].

Pre-radiotherapy material was stained from 106 patients who had definitive

prostate radiotherapy. The mean Ki-67 labelling index was 3.2%, and increased in high grade and high stage disease. A labelling index greater than 3.5% predicted early biochemical failure [90].

A further study of 42 patients with prostate cancer, both capsule confined and extra capsular, demonstrated that Ki-67 as a measure of cell proliferation could predict recurrence in both groups in a multivariate analysis [338]. A later study confirmed these findings[197].

In a series of 55 patients who had received radical radiotherapy and been followed up for a mean of 6.8 years, residual cancer was found in 67% (37/55) of patients, of these 64% (23/36) had active tumour cells as determined by Ki-67 staining. These authors concluded that the majority of patients with tumour on their post radiotherapy biopsies had viable tumour[228].

A study comparing pre radiotherapy biopsies with post radiotherapy biopsies in 13 patients demonstrated an increase in Ki-67 score, between the pre and post biopsies. The score increasing from a mean of 5.6 to 10.5 in those patients that relapsed [142].

3.83 Ki-67 expression and outcome in patients treated with androgen withdrawal.

A study of 153 unselected patients with prostatic carcinoma demonstrated that Ki-67 score was related to poorly differentiated tumour, and a high Ki-67 score was related to metastatic disease and poorer survival. In particular if patients with metastatic disease were stratified according to their Ki-67 scores after androgen withdrawal those with scores > 1% had a poorer outcome [159].

A study of 190 treated patients with prostate cancer with a mean follow up of 12.9 years, demonstrated that Ki-67 was significantly related to stage and grade. In a multivariate analysis Ki-67 expression had no independent predictive value in organ confined disease but was an independent prognostic factor over the entire cohort[1].

3.84 The effect of androgen ablation on Ki-67.

A study of 18 patients who had biopsies before and after androgen ablation showed a decrease in Ki-67 score after androgen ablation[414]. A further study comparing 28 patients who had received neoadjuvant androgen ablation with 51 patients who had not, demonstrated that androgen ablation leads to a significant decrease in MIB-1 index i.e. 7.5% compared to 14.4% respectively[394].

3.85 Ki-67 and PIN.

In a heterogeneous series of 35 patients the Ki-67 labelling index increased in a stepwise progression when comparing benign tissue, with PIN and cancer [187]. This has been confirmed by others [160;275;379;393].

Table20: The relationship between Ki-67 (MIB1) expression and outcome in patients with radical prostatectomy.

Ref.	Year of pub	Type of specimen	No of cases	No localised	No locally invasive	No mets	Method	Results/Conclusions
[30]	1996	Radical Prostatectomy follow up 1-9 yr.	180	72	107	1	IHC	18 pts had low MIB1 expression. Significant correlation between MIB 1 score and nuclear grade, Gleason score, pathological stage. Low MIB1 score correlated with low chance of recurrence.
[207]	1996	Radical Prostatectomy follow up 1-15 yr.	137	44	93	34	IHC	Significant correlation between recurrence and Ki-67>7.5%.
[15]	1999	Radical Prostatectomy follow up 3yrs	29	39	?	8	IHC	High MIB1 expression associated with recurrence, and an independent prognostic factor in a multivariate analysis.
[403]	2000	Radical Prostatectomy follow up 0-17 yr.	92	65	27	6	IHC	High MIB1 expression associated with recurrence, but was not an independent prognostic factor in a multivariate analysis.
[155]	2001	Radical Prostatectomy median follow up 56 months.	104	32	72	7	IHC	High MIB1 expression associated with recurrence, and an independent prognostic factor in a multivariate analysis.
[84]	2002	Radical Prostatectomy	24	13	11	0	IHC	High Ki-67 expression in cancer.
[177]	2005	Radical Prostatectomy	52	14	38	0	IHC	20/52 progressed. 16 Ki67+ relapsed and 4 Ki67-, Ki-67 predicted relapse.
[376]	2005	Radical Prostatectomy	40	6	12	22	IHC	High Ki-67, predicted progression, and metastatic disease.

Table21: The relationship between Ki-67 (MIB1) expression and outcome in patients with radical radiotherapy.

Ref.	Year of pub	Type of specimen	No of cases	No localised	No locally invasive	No mets	Method	Results/Conclusions
[228]	1996	Biopsies	55	?	?	?	IHC	Post DXT, there was still active proliferation.
[338]	1997	Biopsy/TURP	42	30	12	0	IHC	18/23 patients with recurrence had high Ki-67 scores, compared to 6/19 with no recurrence.
[142]	1998	Biopsy	13	13	0	0	IHC	Comparing pre-treatment with post treatment biopsies in radiation failures showed an increase in Ki-67 from 5.6 to 10.5
[197]	1999	TURP	42	35	7	0	IHC	The treatment failures had a significantly higher Ki-67 score compared to the responders.
[90]	2002	Biopsy/TURP	106	74	32	0	IHC	Ki-67>3.5% predicts treatment failure.
[225]	2004	Biopsy	108	31	77	0	IHC	A staining index of greater than 3.5% was associated with a risk of developing local progression, and disease specific mortality.

Table 22: The relationship of Ki-67 to outcome in patients treated with androgen withdrawal.

Ref.	Year of pub	Type of specimen	No of cases	No localised	No locally invasive	No mets	Method	
414	1995	Serial biopsy	18	3	6	9	IHC	Androgen ablation causes a decrease in Ki-67.
1	1997	TURP/Biopsy	190	106	84	66	IHC	Ki-67 increased with grade and stage. In a multivariate analysis Ki-67 staining was an independent predictor of outcome.
370	1997	TURP mean follow up 71 months.	125	89	16	15	IHC	Ki-67 increased with grade and stage. In a multivariate analysis Ki-67>3% was an independent predictor of outcome.

Chapter 4:

Aims

- To determine the relationships between the volume of high grade prostatic intraepithelial neoplasia, cancer and benign tissue.
- To determine the relationship between serum PSA and the volume of high grade prostatic intraepithelial neoplasia, cancer and benign tissue.
- To determine using immunohistochemical techniques the levels of p53, bcl_2, bax, and Ki-67 in formalin fixed benign, malignant and tissue with PIN.
- To determine using immunohistochemical techniques the effect of androgen ablation on the levels of p53, bcl_2, bax, and Ki-67 in formalin fixed malignant tissue.

Chapter 5: Materials and Methods.

5.1 Methods

This study was divided into two parts; the first part was a prospective assessment of tumour volume, PIN volume, and total gland volume. This was carried out in 76 radical prostatectomy specimens. The second part was a retrospective assessment of the expression of p_{53}, bcl_2, bax, and Ki-67. This was carried out on paraffin fixed specimens identified from 97 radical prostatectomy specimens.

All radical prostate specimens were obtained from patients undergoing radical retro pubic prostatectomy. The patients preoperatively were staged with clinical examination, serum total PSA (Hybertech Assay), transrectal ultrasound guided sextant biopsy, CT scan and bone scan. A single surgeon (A V Kaisary) carried out the operations and a single pathologist carried out all the initial histopathological assessment (M Jarmulowicz).

The specimens were prepared as follows.

5.2 Fixation

Some authors advocate cutting the prostate into slices fresh and then placing in fixative[344]; we have not found this a satisfactory technique. It is difficult to cut the prostate fresh and get consistent slices. In addition the tissue is friable and distorted. This leads to curling of the edges. Others believe in inking the fresh prostate and then fixing it whole [343]. We feel the best results are achieved if the latter approach is adopted. After weighing, the whole specimen is left to fix in 10% buffered formaldehyde for at least 72 hours. The specimen is not incised to speed penetration of the fixative, as this will distort the subsequent slices.

After fixation the specimen is measured. The margins are painted with Indian ink. After painting the specimen is briefly immersed into Bouin's fixative, which instantly fixes and hardens the Indian ink, so minimising the spread of ink onto the cut surface on slicing. The specimen is then cut.

5.3 Cutting the specimen

The specimen is cut with a slicer. The machine setting is calibrated, using any available fixed tissue, to deliver slices which are 3.5 mm in thickness. Our experience of these machines is that towards the end of the cut, the specimen slice is pulled away producing a curved slice and distorted surgical margin at

that point. To prevent this, the specimen is almost cut through, and then withdrawn from the machine and the cut completed using a disposable microtome blade attached to a disposable handle.

There are various opinions on the best way to tackle the apical slice. In our laboratory the apical slice is further cut in the saggital plane and then sliced[343].

5.4 Scanning the slices

Maintaining the correct orientation, both left / right and apex to base, the slices of prostate are laid onto an A4 sheet of transparent celluloid. The surface of the slices to be cut to produce the microscope section is laid face down onto the celluloid. The slices, together with a graduated rule, are then placed in a standard flatbed scanner. The specimen is covered with a white A4 sheet of paper, both to protect the lid of the scanner and to improve the background. As the lid will be three millimetres above the glass the background will have a green hue due to reflection from the lid.

Select the image size to be scanned as A4. This will make subsequent printing at real size easier. Scan the image at 180 dpi with printing at 720 dpi (Figure 14).

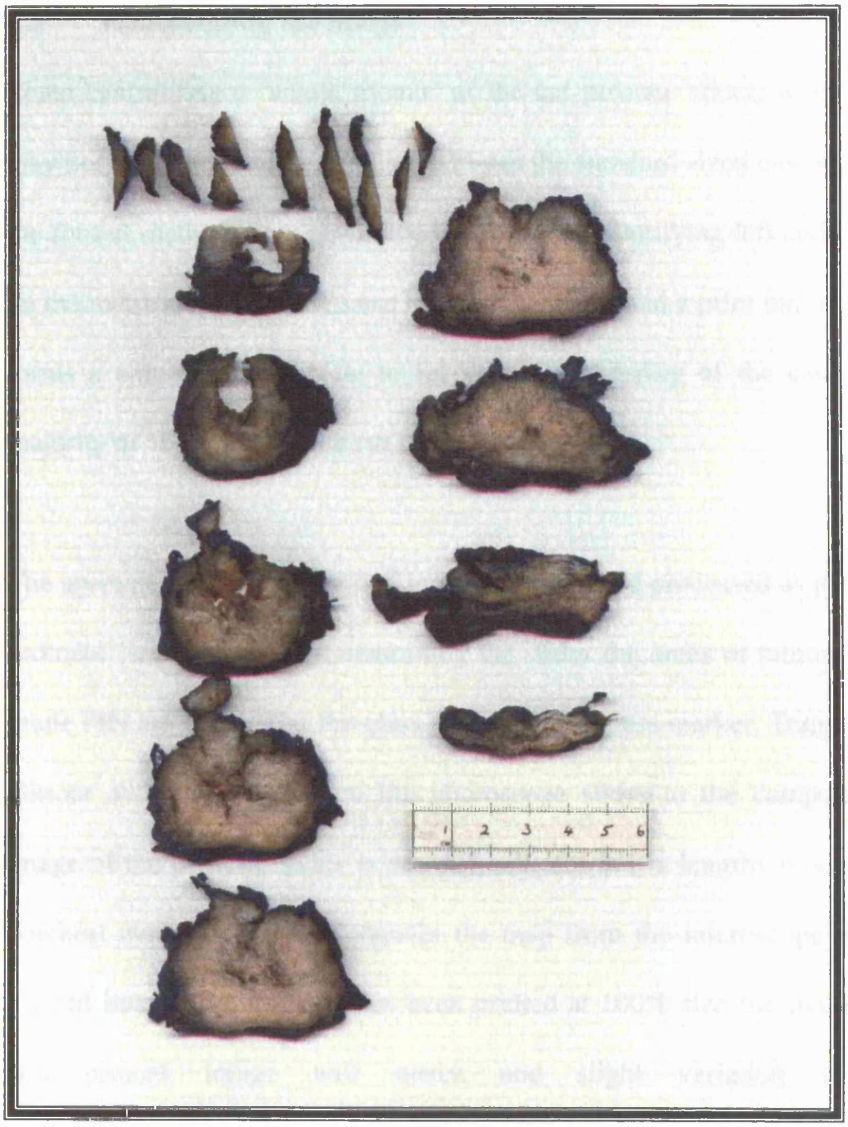

Fig 14:After fixation the prostate is sliced and scanned.

5.5 Manipulating the image

Some centres use a 'whole mount' of the cut prostate slices, whereas others may not have the facilities and have to use the standard sized cassettes. Using the former method there should be some way of identifying left and right sides on the microscope slide. We use standard cassettes and a print out of the image forms a convenient template to record the numbering of the cassettes. The majority of slices need to be cut into four.

The specimen is then embedded in paraffin wax and processed as per standard protocol (see later). When examining the slides the areas of tumour and high grade PIN are marked on the glass slides using a glass marker. Transferring the tumour and PIN maps from the microscope slides to the computer bitmap image of the prostate slices is not difficult, nor is it a lengthy procedure. The quickest method is to first transfer the map from the microscope slides to a printed image. If the image has been printed at 100% size the tissue sections and printed image will match and slight variation to section shrinkage/stretching does not produce any significant difficulties. The printed image can be traced over the slide using a light box; the latter is the easier and more accurate (fig 15).

Fig 15: Areas of PIN and cancer.

To achieve the final digitalised image the hand coloured printed hardcopy is re-scanned and overlaid onto the original image. This is straightforward as the latest version of software allows images to be manipulated as a series of separate image layers. The re-scanned image is slightly smaller than the original, because of the small margin introduced by the printer, but this is easily corrected using the resize command of the software, so that the two images are of the same size. The overlaid image, in a separate layer, is set at 50% transparency so that it can be moved to perfectly place it over the original image. A digitalised pad with a pen is used to facilitate this stage rather than a standard mouse. For clarity the marked tumour and PIN on the re-scanned layer is traced in the appropriate colour (Red = cancer, Blue = PIN), each in a separate image layer. The final image can then be printed (figure 16). The patient identification, slice labels and any comments can readily be inserted onto the image using the text facility of the image software. For convenience it is best to have separate image layers for prostate slices, patient/specimen identification, labels and comments, tumour and PIN. Using Paint Shop pro (version 6) the image saved as separate layers is approximately.

This technique provides a high quality image of the cut surface of the prostate, with an overlaid map of tumour and PIN.

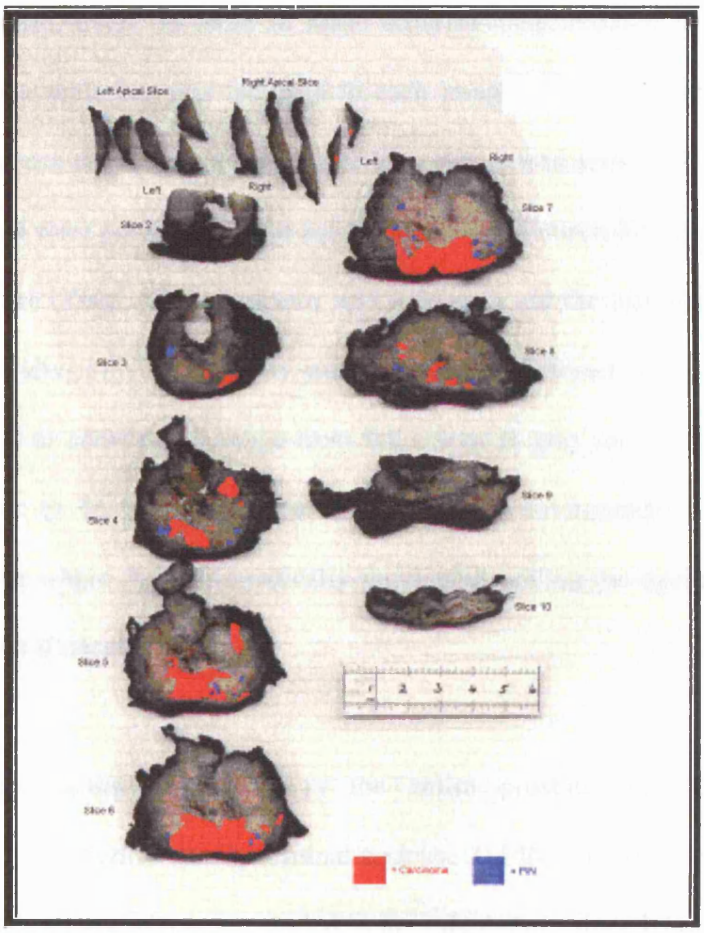

Fig 16: Composite image of prostate.

5.6 Calculation of total prostate, PIN and tumour volume

The acquired images of prostate slices were transferred to the image analysis program using proprietary software (Sigma Scan Pro, version 6.0 by Jandel Scientific, UK). In order to make accurate measurements of prostate gland areas; a scale bar was included in each image. The image analysis program would use the scale bar for calibration. Area measurements of the regions of interest were performed semi-automatically on Kontron KS400 image analysis software (Zeiss, UK). A macro was written to aid the use of this programme (Appendix 1). The macro was designed to interact with Paint-Shop-Pro images to convert the image from full colour to grey scale and then allow the images to be transported out to the KS400 environment. The regions of interest where then automatically segmented, giving the operator a choice to interact if necessary.

Regions measured included: 1) the entire prostate excluding the slices containing predominantly seminal vesicles, 2) PIN, and 3) cancer. The area of the apical cone was not included in the calculation, since it was difficult to be certain of its exact thickness and so the volume it represented. After the areas had been calculated they were multiplied by the shrinkage factor to correct for any changes in volume during processing of the specimen. All measurements were transferred to Microsoft Excel for statistical analysis.

5.7 Shrinkage factor

Shrinkage factors have been calculated by a number of authorities, and vary from 1 to 1.5[301;344]. It is important to calculate it for your specific lab. In addition different labs use different techniques to fix the prostate with different fixatives and different protocols for the processing of the prostate[343]. Some labs cut the fresh prostate into slices and then fix the gland in slices[343] where as others, ours included, fix the whole gland and then cut it into slices.

In our lab we do not have the resources to process the prostate slices as whole mount so as described previously we cut them into portions that will fit into our cassettes. Taking all this into account we decided to calculate the shrinkage fraction for our lab rather than accept one that may not be applicable to our institution.

The first problem is how to accurately the measure the volume of the prostate. Since prostates are an irregular shape and often compressible it is difficult to get reproducible measurements with a ruler. Therefore, the fresh prostates were taken from the operating theatre, loose tissue was removed and they were weighed and immersed in water and the volume of water displaced weighed. The measurement of the volume was repeated. The prostates are then placed in the fixative, 10% buffered formaldehyde for at least 72 hours.

Just prior to slicing the prostates, the measurement of volume and weight was repeated. This gave us the shrinkage factor for the first stage. This method of measuring changes in the volume takes into account any contraction that occurs in the urethral cavity whereas if simply the maximal linear measurement was taken before and after fixation this would not have been taken into account[343]. However the volume obtained also includes the seminal vesicles which may have different tissue characteristics with respect to fixation and processing. We did consider removing the vesicles prior to measuring the volumes, but this would have meant considerable dissection of the specimen prior to histopathological assessment of the specimen, and it was felt this would not be in the best interest of the patient. In addition other authorities have included the seminal vesicles when calculating the shrinkage fraction[301].

Since the slices are now scanned, this factor can be used for determining the total volume of the gland from the images. Then the slices are divided into convenient sections for placing in the cassettes; initially we attempted to use linear measurements with a vernier calliper. However this was not reproducible and there was considerable inter and intra user variation. Therefore this part of the procedure evolved, and now we scan the prostate slices whole after the fixation in formalin and then the processed slides stained with haematoxylin and eosin were scanned (fig 16).

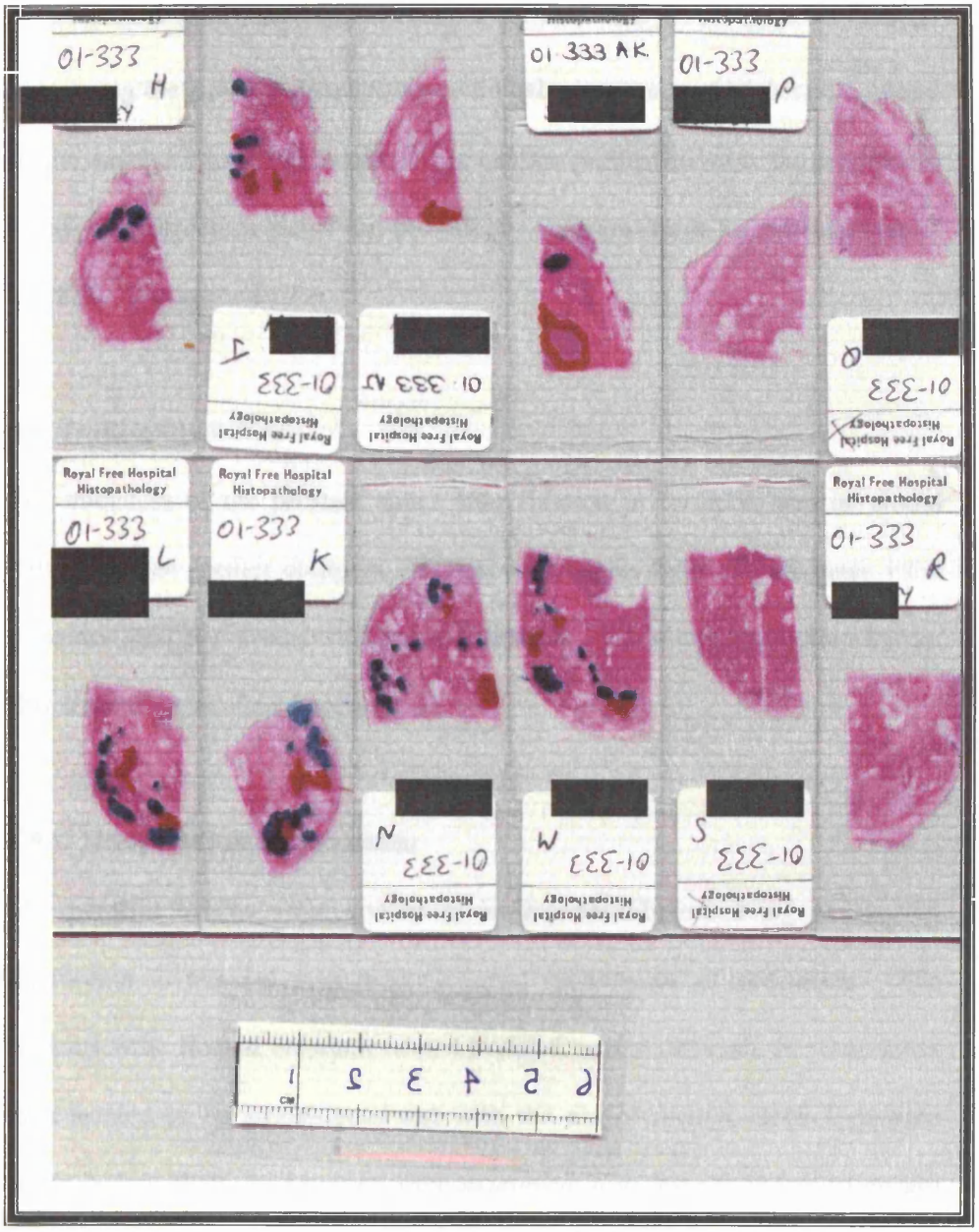

Fig 16:H&E stained slices for comparison with slices from fig 14.

Thus using the KS-400 software we could calculate the area of these slices and so the second correction factor. This can be multiplied with the previously calculated correction factor can provide an accurate result for prostate cancer and PIN. See appendix2.

5.8 Slice thickness.

The thickness of the prostate slices after fixation in formalin was measured with a pair of Venier callipers. Consecutive slices from six prostates were measured and the average thickness calculated. This was multiplied by the shrinkage fraction. See Appendix 3.

5.9 Preparation of specimens

The paraffin blocks were cooled in an ice bath before using a standard microtome to cut 3-4 micron thick serial sections of all specimens. The sections were floated on water heated to 45° C in a water bath. In some cases they needed to be straightened out with the aid of methyl alcohol covered slides before floating. Sections were examined with the naked eye to ensure that a cross section of the whole block was obtained. Each slide usually contained one piece of serial section from, the same block. The sections were then mounted on to APES coated microscopic glass slides (Appendix No. 4) to avoid tissue loss during micro waving. The slides were immediately labelled

using, a lead pencil, with their respective pathology number which consists of a 7 digit code (identical to number on block). The upper left corner of the frosted part of the slide was consistently used to ensure correct orientation when handling them. Slides were then transferred into racks and left for 24 - 48 hours in a warn room at 37^0 C to dry.

5.10Dewaxing.

Prior to staining the slides had to dewaxed. This was carried out as per appendix 5. Then the specimens could be stained with haematoxin and eosin or with the appropriate antibodies.

5.11Staining methods

There are four main methods of immunoperoxidase staining that can be used to localise cellular antigens. The direct, indirect, PAP, and streptavidin-biotin methods. Each may have certain advantages and disadvantages which have been evaluated in our laboratory; we use the streptavidin-biotin method. The streptavidin-biotin technique lends itself to the localisation of numerous antigens in a variety of specimens including paraffin sections, cryostat sections, smears, imprints and cytospins. For a specific antigen it can be determined what type of cells produce this substance in normal and neoplastic tissue, levels of the substance produced, and the determination of tumour cell

differentiation.

Immunoperoxidase staining involves the use of antibodies and the enzyme peroxidase. Peroxidase is commonly used for several reasons:

1. Its small size will not hinder the binding of antibodies to adjacent sites.
2. It is easily obtainable in highly purified form so that the chance of contamination is minimised.
3. It is very stable, and therefore will remain unchanged during manufacture, storage and application.
4. Only small amounts are present in tissue specimens, and this endogenous peroxidase activity is easily quenched.
5. There is a wide availability of chromogens, which can be acted upon by peroxidase to form a coloured end product that will precipitate at the site of the antigen to be localised.

5.12 Streptavidin-biotin Method

This method is based on the ability of the egg white glycoprotein avidin to immunologically bind four molecules of the vitamin biotin. Three reagents are used. The first is a primary antibody specific for the antigen to be localised. The secondary antibody, capable of binding to that first, is conjugated to biotin. The third reagent is a complex of peroxidase conjugated biotin and

avidin. The free sites of the avidin molecule allow binding to the biotin on the secondary antibody. The peroxidase enzyme, and therefore the original antigen, is visualised with an appropriate chromagen.

Even though conjugated antibodies are used in this method, the strong affinity of avidin for biotin gives this method greater sensitivity than other conjugated antibody techniques such as the direct and indirect methods. Excellent results can be achieved on fixed, paraffin embedded specimens.

5.13 Staining Procedure

The details of the exact staining procedure are summarised in Appendix No.6.

5.14 Endogenous Peroxidase Activity (Appendix No. 7)

The substrate chromagen reaction used to visualise peroxidase cannot distinguish between the enzyme immunologically localising the cellular antigen, and similar enzymatic activity present in the specimen before staining. This endogenous peroxidase activity is confined mostly to red and white blood cells.

If it is not removed before adding the marking enzyme, positive staining will be observed that is due not to the specific antigen alone, but also due to peroxidase activity already present in the specimen. Removal of endogenous

peroxidase is essential to correct interpretation. This was achieved by quenching the mounted specimens in a solution of 0.5% hydrogen peroxide in methanol.

5.15 Antibodies

The Dako D07 was used for the detection of aberrant p_{53} because this has been shown to be an effective antibody in comparative studies of six antibodies [13], and has been used in our laboratory routinely and by others with good results [10]. Dako M887 was used for bcl_2, SantaCruz Bax N20 for bax alpha, Dako M722 for Ki-67, and Dako A562 for PSA.

5.16 Dilution of antibodies.

Optimally diluted antibodies must be used to achieve meaningful results. The dilution of any one antibody is dependent upon several factors:

- Concentration of specific antibody in the solution as provided by the manufacturer (antibody titre). The more specific antibody molecules per millilitre, the higher the titre and the lower the working concentration.

- A number of substances other than specific antibody are often present in the solution; high levels of contaminating proteins necessitate high dilutions to prevent non-specific background staining.

Generally the longer the incubation time, the more dilute the antibody can be used. In this study, antibody incubation times were held constant after experimental determination of optimal time.

Other considerations such as the choice of dilution buffer, use of a humidity chamber, the specimen fixation and processing protocol, will all affect the dilution used. Optimal dilutions for our laboratory conditions were ascertained for each individual antibody.

When interpreting dilution results, two criteria were evaluated: Specific staining and non-specific background staining. The goal was to achieve the greatest intensity of specific staining with the least amount of background interference. The higher the antibody dilution, the lower the background staining due to undesired protein binding.

5.17 Incubation times

Incubation times, antibody dilutions, non-specific background staining and specific staining intensity are all interrelated. The longer the incubation times the higher the antibody dilution. The more dilute the antibody, the lower the non-specific background stain. The lower the non-specific stain, the greater the contrast with the specific stain, making interpretation easier.

Incubations with antibody solutions were for 60 minutes to 16 hours. When processing several slides at once, there will be a one or two minute difference between the first slide and the last. To achieve consistency processing of slides was staggered so that all specimens undergo identical incubations. The temperature of incubation will also affect antibody dilutions. The optimal temperature is calculated for each antibody.

Haematoxylin and eosin stains were carried out on all the sections. This determined the presence of carcinoma, PIN, and benign prostate tissue. The areas of tumour and PIN were demarcated and then transferred to the computer-generated images.

5.19 Antigen retrieval (micro waving) (Appendix No.9)

The use of formalin fixed paraffin blocks was unreliable before the advent of antigen retrieval techniques such as microwave irradiation or a pressure cooking to reverse some of the effects of fixation (un-fixation). Studies indicate that antigen retrieval provides an extremely successful method for the demonstration of nuclear antigens with formalin fixed, paraffin embedded tissues [351-355;383;384], even after fixation in formalin for as long as 7 days [383]. The use of an antigen retrieval system provided excellent results with minimal background staining, and high contrast between positively stained and negative

cells. Studies for p_{53} using single strand conformational polymorphism analysis of RNA/PCR products and sequencing have shown that using microwave irradiation as an antigen retrieval system detects 93% of mutations between exons 5-8 [413].

For the immunohistochemical studies the slides were microwaved or placed in a pressure cooker as part of an antigen retrieval system. The microwave incubation times had been predetermined (PSA = 5mins, p_{53} = 10 minutes, and bcl_2 =20 minutes). For Ki-67 pressure-cooking has been used successfully in our laboratory as an antigen retrieval system, the time used was 90seconds. For bax no antigen retrieval is required.

5.19 Controls

Certain procedural and reagent controls are essential for the validation of the results of immunoperoxidase staining. Most of these controls centre on the primary antibody to ascertain that positive staining is the result of specific binding to the antigen. After determining the optimal dilution of a new primary antibody, several known positive and negative specimens were stained to certify the antibody's specificity.

Positive control: A specimen, processed identically to the unknown that contains the antigen in question.

Two negative controls: One specimen processed in an identical way except that non-immune serum is used instead of the primary antibody. This serves as a specimen blank since any staining observed is due not to antibody localisation of the antigen but to non-specific protein binding, endogenous peroxidase activity, or non-specific binding of the other antibody reagents.
The second specimen processed in an identical way except that non-immune serum is used in place of both primary and secondary antibodies. This serves to exclude staining that may be due to interaction between the chromogen used 3, 3 Diaminobenzidine Tetrachloride (DAB) and processed tissue (DAB negative control).

All controls underwent the same fixation and processing schedule as the unknown specimen and were processed along with the unknown to assure the accuracy of the results.

Antigen	Clone	Supplier	Antigen retrieval.	Ab Dilution	Incubation time.	Controls
P_{53}	D07	Dako	10 mins microwave	1:50	60 mins @ room temp.	Vulval cancer
Bcl_2	M887	Dako	20mins microwave	1:25	60 mins @ room temp.	Tonsil
Bax-alpha	Bax N20	SantaCruz	0	1:800	16hrs @4°C	Breast
Ki-67	M722	Dako	90s in 2l Citrate buffer in a pressure cooker.	1:500	60 mins @ room temp.	Tonsil
PSA	A562	Dako	5 mins microwave	1:800	60 mins @ room temp.	Benign prostate

Table23: A summary of the antibodies used the incubation times, and the controls.

5.20 Principles of Interpretation of immunostaining

The most difficult aspect of immuno-staining is in the correct evaluation of the finished product. The amount of chromagen precipitated, and therefore the intensity of the reaction has been found to be proportional to the amount of antigen present. Not all cells contain the same amount of antigen, and therefore stain with varying intensity. Non-antigen containing cells and cell components are contrasted by a counter stain (methylene blue) which makes distinguishing the two easier.

Specific staining is localised in cells, non-specific background staining is usually found in collagen and connective tissue. Interpretation is basically a comparison of the specific and non-specific staining pattern and intensity, if any, of the specimen with that of the control. The source of the tissue, antigen characteristics and the procedure used must be taken into account for proper evaluation.

Several artefacts maybe found in specimens, which may produce staining and interfere with correct interpretation. They are mostly not confined within cells, but are spread randomly across the specimen. Any staining that occurred on the very edge or along knife marks is often non-specific and can be ignored. Positive staining of necrotic crushed or cells that have undergone autolysis and haemorrhagic tissue, is always regarded as non-specific. Only the staining pattern of viable cells is considered for interpretation.

5.21 Non-specific Background Staining

Positive staining of a specimen that is not a result of antigen antibody binding is termed non-specific background staining. The most common cause is attachment of protein to highly charged collagen and connective tissue elements of the specimen. Antibodies are proteins. If the first protein solution applied to the tissue is the primary antibody, it can be non-specifically adsorbed to these charged sites. The secondary antibody can still bind to the

primary and the peroxidase colour reaction will occur. Positive staining of these sites is due not to localisation of the tissue antigen, but to non-specific antibody attachment to collagen and connective tissue, and neurones and ganglion cells for bcl_2.

Other causes of non-specific background staining such as inappropriate antibody dilutions incomplete removal of paraffin, improper rinsing of slides and incorrect substrate incubation can be minimised by careful and accurate technique.

5.22 Scoring of immunostaining

All samples were scored firstly as positive or negative staining, secondly by assessing the intensity of staining, thirdly by assessing the proportion of cells stained. In carcinoma and PIN this grading was confined to only regions of interest within the specimen. The areas of PIN and cancer within each specimen were initially identified with H&E.

Cases were considered positive if any of the tumour cells manifested positivity. Intensity was scored on a four point scale: no staining = 0; weak staining = 1; moderate staining = 2 and intense staining = 3. Assessment of intensity of staining was always referenced to a standard positive control, stained at the time and under the same conditions as the test case.

The proportions of cells staining, for KI-67 in particular, were scored as a percentage of cells staining positive against total number of cells per high power microscopic field (x 400). All histopathological assessment was confirmed two histopathologists, Dr Michael Jarmulowicz, Consultant Histopathologist at the Royal Free Hospital, and Dr Tara Walker, Consultant Histopathologist at Queen Alexandra Hospital, Portsmouth. There was agreement in most (>90%) of cases with regard to the intensity and pattern of staining between the two pathologists. In the sections in which the scores differed, we agreed a common result. The percentage of positive cells within a section was estimated by 'eye-brain' co-ordination.

5.23 Background staining

Background staining was minimal or absent in most sections and did not therefore interfere with assessment. The p_{53}, bcl_2 and MIB-1 antibodies achieved particularly specific staining with an absence of background staining. The bax had some diffuse background staining.

5.24 Staining patterns

The criterion for a positive reaction was nuclear staining for p_{53} and Ki-67 and cytoplasmic staining for bcl_2, bax alpha.

5.30 Statistics.

The data obtained from this study was tabulated in an excel spreadsheet. The raw data is presented in Appendix 10 and 11. The data was assumed not to follow a normal Gaussian pattern, and therefore treated as a non-parametric data set. Analysis designed for parametric data can not be applied to non-parametric data, but non-parametric analysis can be applied to parametric and non-parametric data to give valid results. Therefore the use of non-parametric tests would maintain the statistical stringency of the analysis.

The stastical package for social science (SPSS) 10.1 was used for data analysis. The analysis of variance (ANOVA) was used calculate the relationship between tumour, PIN, and benign tissue volume and serum PSA. The data obtained from the staining of the slides was also assumed to be non-parametric, so the Mann-Whitney U test was used to compare the relationship between the slides stained.

Assistance and guidance about statistical method was sought from Richard Morris (Department of Statistics, The Royal Free Hospital), David St George (Department of Statistics, The Royal Free Hospital) and Roger A'Hern (Department of Statistics, The Royal Marsden Hospital).

Chapter 6: Results

The results obtained during this piece of work have been divided into two sections. The first set of data was obtained from the morphometric analysis of 76 radical prostatectomy specimens. This was carried out prospectively. In summary the whole radical prostatectomy was fixed in 10% buffered formaldehyde for 72 hours. The specimen is then weighed and inked, slices are prepared at intervals of 3.46mm, the slices are scanned and the image obtained is used as a template. The slices are then cut into four and placed in a standard cassette. Slides are prepared from the cassettes and haemotoxylin and eosin stain is used to determine the areas of interest. This is transcribed onto the initial template. A shrinkage fraction for the template is 1.03, and for the areas of cancer, and PIN is 1.22.

The data is presented in graphical form wherever possible. The raw data is available in appendix 10.

6.1: Results 1: Morphometric Studies.

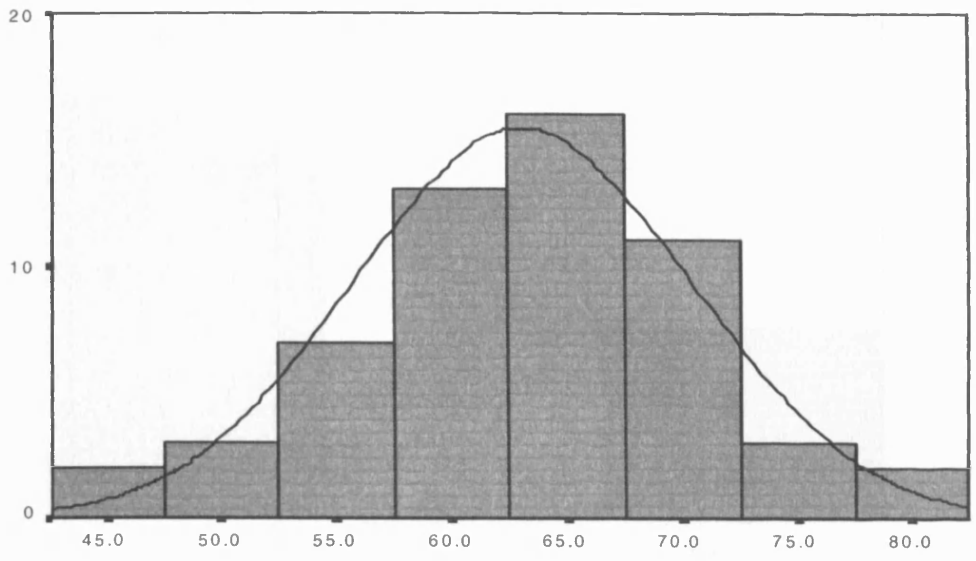

Fig 18:The age of the patients.

This graph represents the age distribution of the 76 patients whose prostatectomy specimens were used to investigate the relationship between serum PSA, cancer volume and PIN volume. The mean age of the patients at the time of surgery is 63.07 +/- 7.4 (SD), with a range of 46 to 78 years.

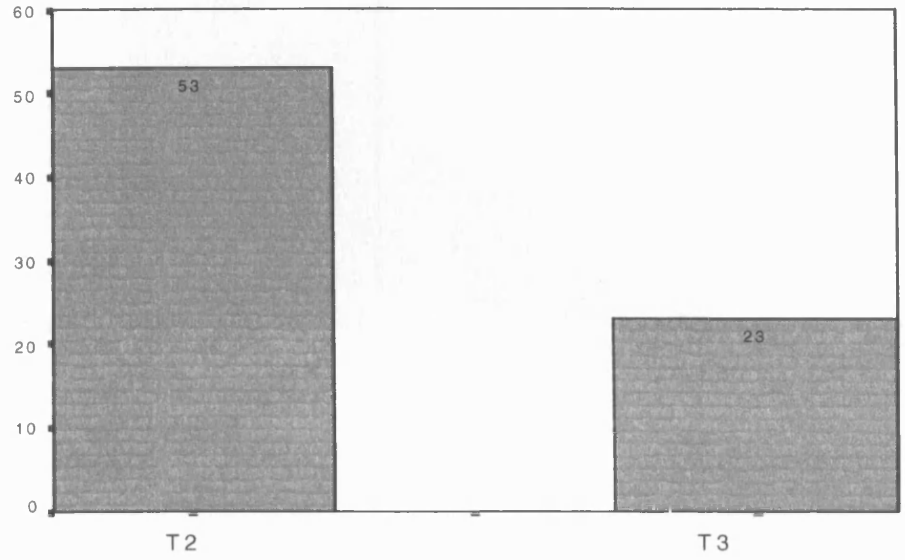

Fig 19:Stage of radical prostatectomy specimens.

This graph represents the stage distribution of the radical prostatectomy specimens obtained from the 76 patients whose prostatectomy specimens were used to investigate the relationship between serum PSA, cancer volume and PIN volume. The stage of specimens was assigned using the International Union against Cancer (UICC) TNM Classification of malignant tumours, 6th edition, published in 2002.

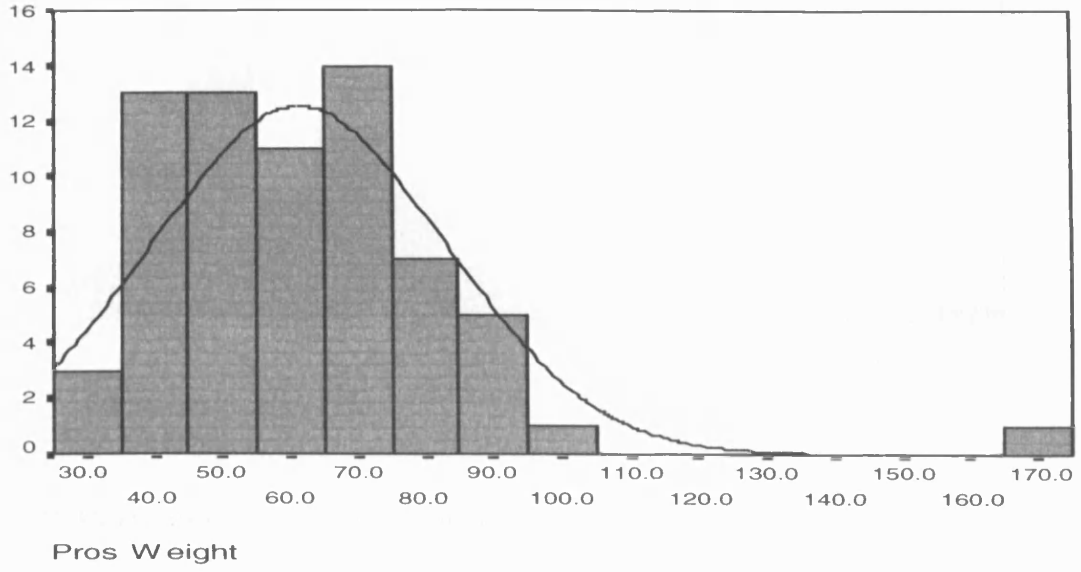

Fig 20:Distribution of weight of gland.

The specimens were obtained fresh from the operating theatre. All loose tissue was removed. The specimens were then directly taken fresh to the histopathology department and weighed. The mean fresh weight is 61.3 g +/- 21.6 (SD) with a range of 26 to 173 g.

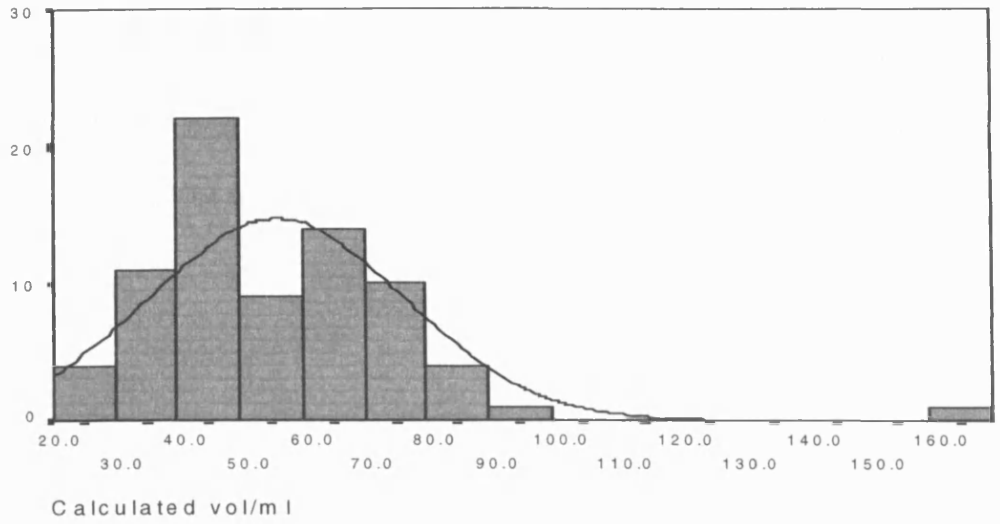

Fig 21:Distribution of volume of glands.

Prostatectomy specimens are an irregular shape and therefore linear instruments can not be used to calculate their dimensions. The volume of the prostatectomy specimen was calculated by obtaining the fresh specimen, removing any loose tissue, and fixing it in formaldehyde. The slices were cut at 3.5mm intervals. They were then scanned using Paint Shop Pro (version 6.0). The scanned images were transferred using Sigma Scan Pro (version 6.0 by Jandel Scientific, UK). This made the images into a format accessible to image analysis software. The software used was Kontron KS400.

The regions of interest could then be identified and measured. In order to measure the volume of the gland, the area of all the slices except the apical slices were measured. This was multiplied by the thickness of the slices and the shrinkage fraction, to obtain the volume of the entire gland. There is an

assumption made that the prostate specimen is of uniform density. The mean calculated volume of the prostatectomy specimens is 50.9 mls +/-20.6 (SD), with a range of 19.4 to 155.5 mls.

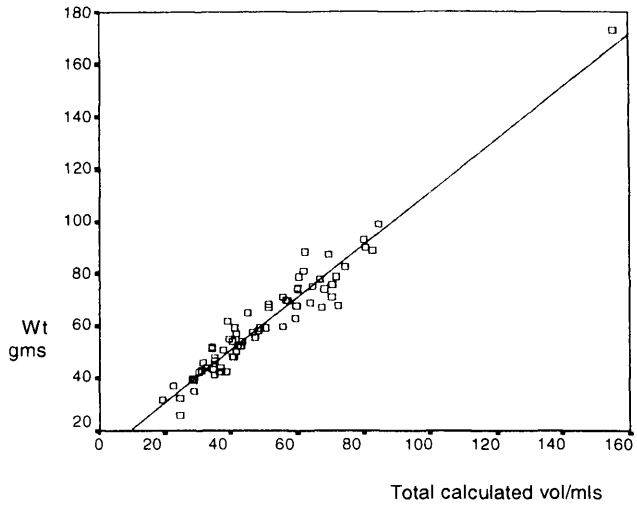

Fig 22:Correlation between calculated volume and weight of gland (Rsq = 0.985).

The data obtained from the weight and the volume of the specimens was used to calculate a correlation coefficient. SPSS software was used and analysis of variance (ANOVA) was used to calculate the correlation coefficient.

There is good correlation between the volume of the specimens and the weight of the gland. This is reassuring and further supports using the scanned images to accurately calculate the volume of the gland. The majority of human tissue is composed of water so the density obtained from this calculation is close to 1g/ml.

The line does not cross the 0 on the x and y-axis simultaneously. This could be due to the volume of the apical slices not being included in the volume calculations.

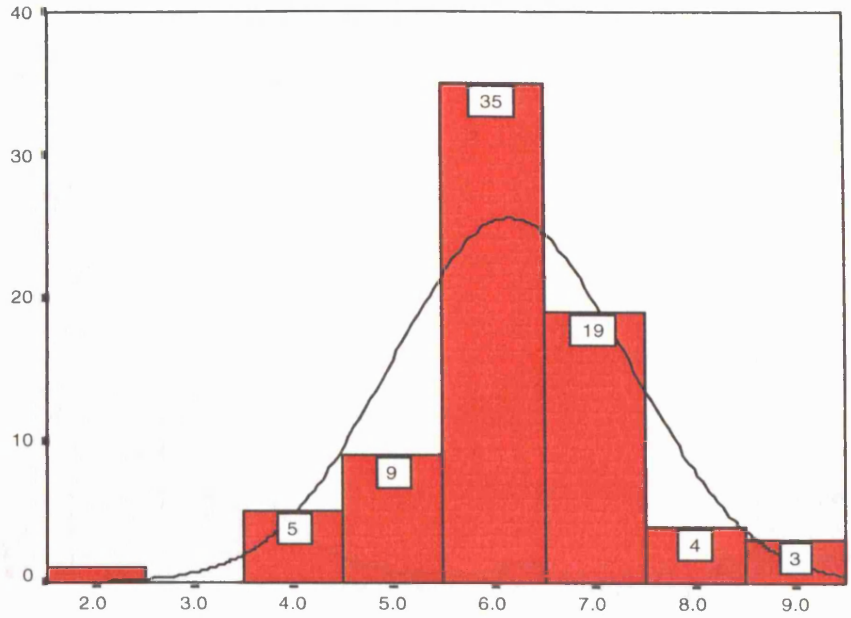

Fig 23:Distribution of Gleason score

The Gleason score is the sum of the two most common Gleason grades. The Gleason grade is determined by the architecture of the prostate cancer[131;250]. The Gleason score will affect the management options available to the patient, for example patients with a Gleason score of seven or above are rarely offered surveillance. The Gleason score also has prognostic significance, in this series the mean Gleason score is 6.2 +/- 1.2 (SD) with a range of 2 to 9. This is in keeping with the results from other published series.

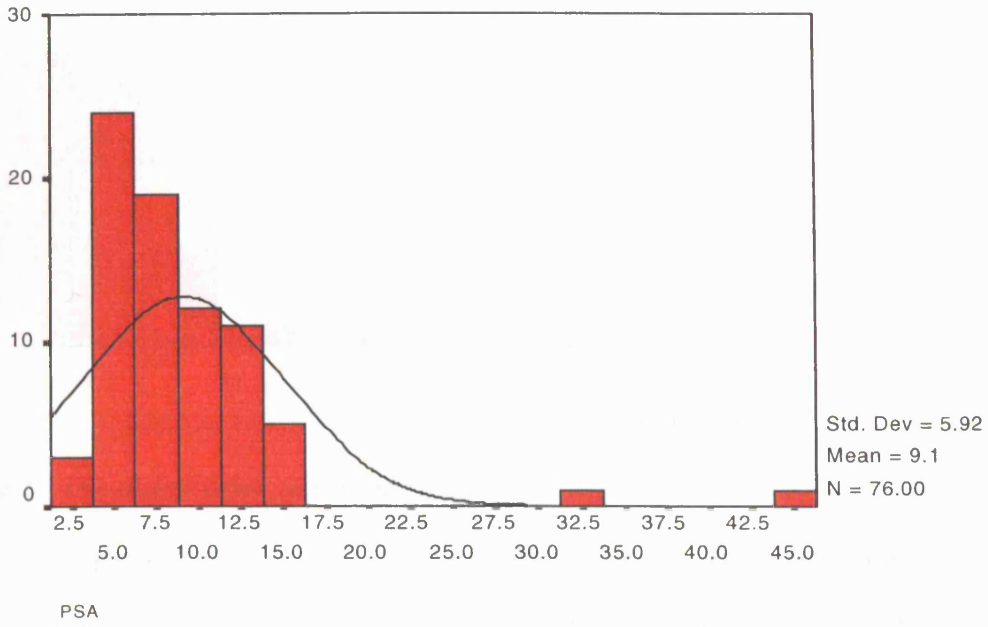

Fig 24:The distribution of PSA (ng/ml) in the 76 patients studied for morphometric analysis.

The blood sample for the PSA was taken the night before the operation. The hybertech assay was used for PSA. The range of PSA was 3 to 45 ng/ml, with a mean of 9.1+/- 5.9 (SD), and a median of 5.5 ng/ml.

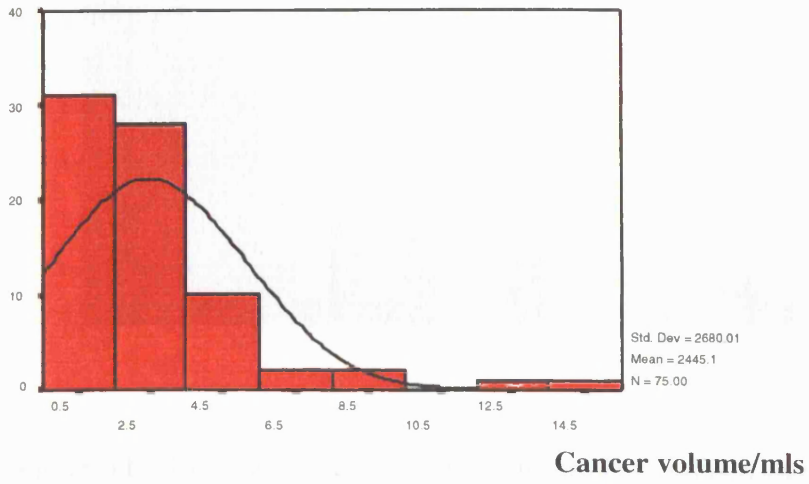

Fig 25:The distribution of cancer volume.

The fresh prostate was fixed for 72 hours in formaldehyde. The specimen was then sliced at 3.5-mm intervals; the slices were then scanned using Scan Pro (version 6.0). Thus preparing a template. The prostate slices were placed in cassettes and embedded. Slides were prepared and stained with haematoxylin and eosin. The areas of cancer were identified. This was transcribed on to the template in red. The scanned images were transferred using Sigma Scan Pro (version 6.0 by Jandel Scientific, UK). This made the images into a format accessible to image analysis software. The software used was Kontron KS400. The regions of prostate cancer were identified and measured. In order to measure the volume of the prostate cancer, the area of all the areas of cancer except the apical slices were measured. This was multiplied by the thickness of the slices and the shrinkage fraction, to obtain the volume of the cancer. The range of the prostate cancer was .09 to 15 mls with a mean of 2.5 +/-2.7 (SD).

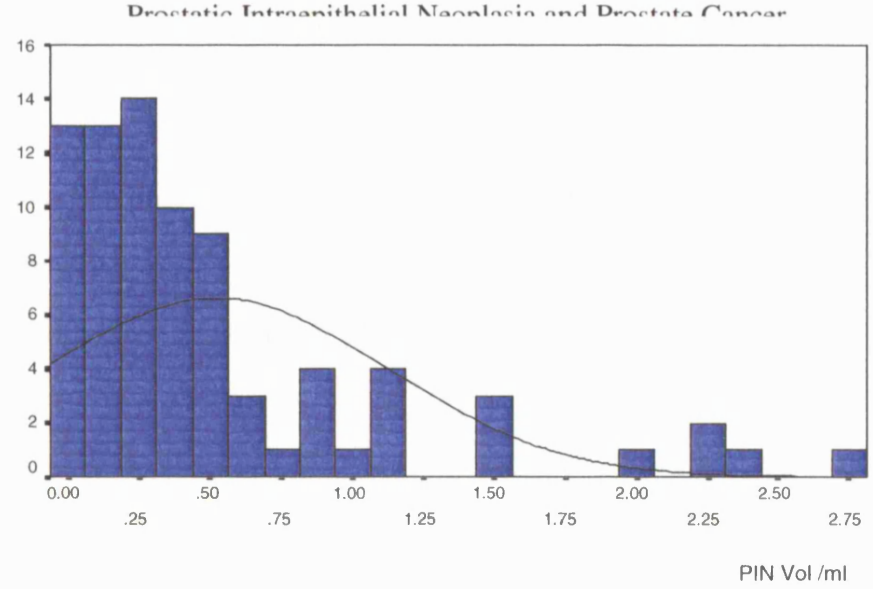

Fig 26:The distribution of PIN volume in the 76 prostates studied.

The fresh prostate was fixed for 72 hours in formaldehyde. The specimen was then sliced at 3.5-mm intervals; the slices were then scanned using Scan Pro (version 6.0). Thus preparing a template. The prostate slices were placed in cassettes and embedded. Slides were prepared and stained with haematoxylin and eosin. The areas of PIN were identified. This was transcribed on to the template in blue. The scanned images were transferred using Sigma Scan Pro (version 6.0 by Jandel Scientific, UK). This made the images into a format accessible to image analysis software. The software used was Kontron KS400. The regions of PIN were identified and measured. In order to measure the volume of PIN, the area of all the areas of PIN except the apical slices were measured. This was multiplied by the thickness of the slices and the shrinkage fraction, to obtain the total volume of PIN.

The range of PIN measured was 0 to 2.7 mls with a mean of 0.53 +/-0.6 (SD) mls. The area of the prostate taken up by benign tissue was calculated by

subtracting the volume occupied by PIN and prostate cancer from the calculated volume of the whole gland. The range of volume for benign gland was 47.5 mls with a range of 13.3 – 153.4 mls.

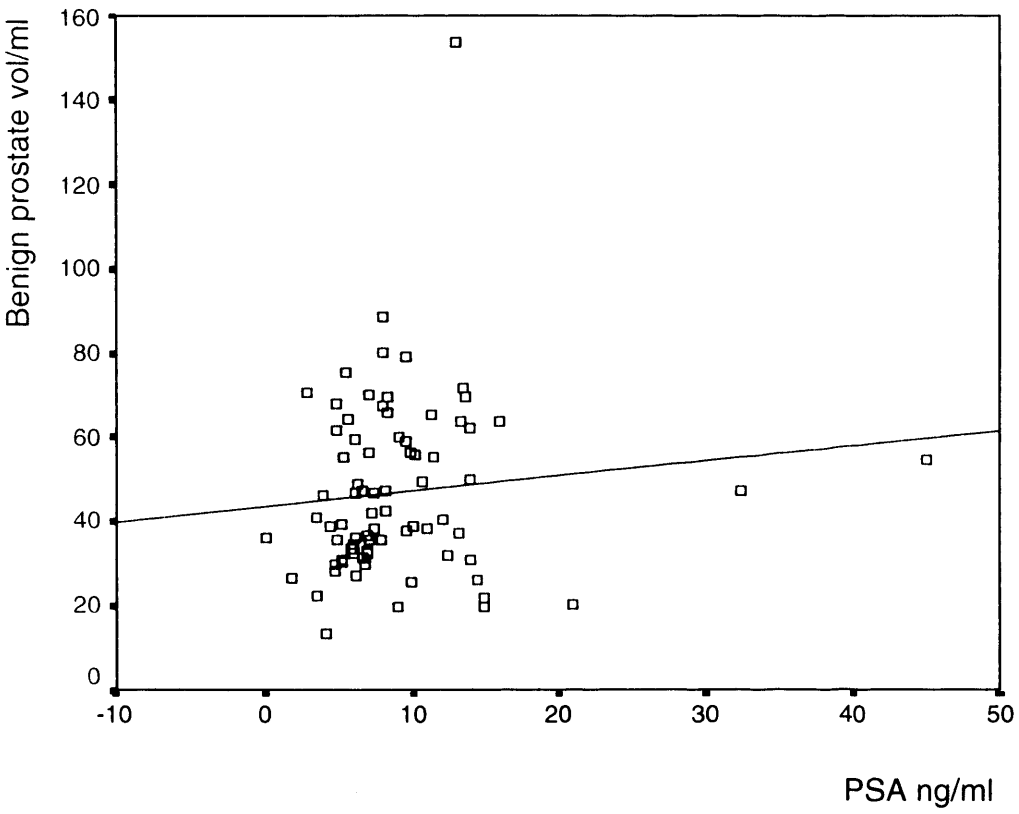

Fig 27: Relationship between serum PSA and benign volume of prostate.

The volumes were calculated using the scanned images, multiplying by the slice thickness and making a correction for the shrinkage fraction. The benign volume of the gland was calculated by subtracting both the volume of PIN and cancer from the total volume of the gland i.e. total prostate volume – (volume of PIN + volume of prostate cancer). The volume of the benign tissue was plotted against the serum PSA (ng/ml) using SPSS10.1 software. The scatter chart generated correlation (spearman rho=0.24). The correlation between the serum PSA and the volume of benign prostate is significant (p=0.05).

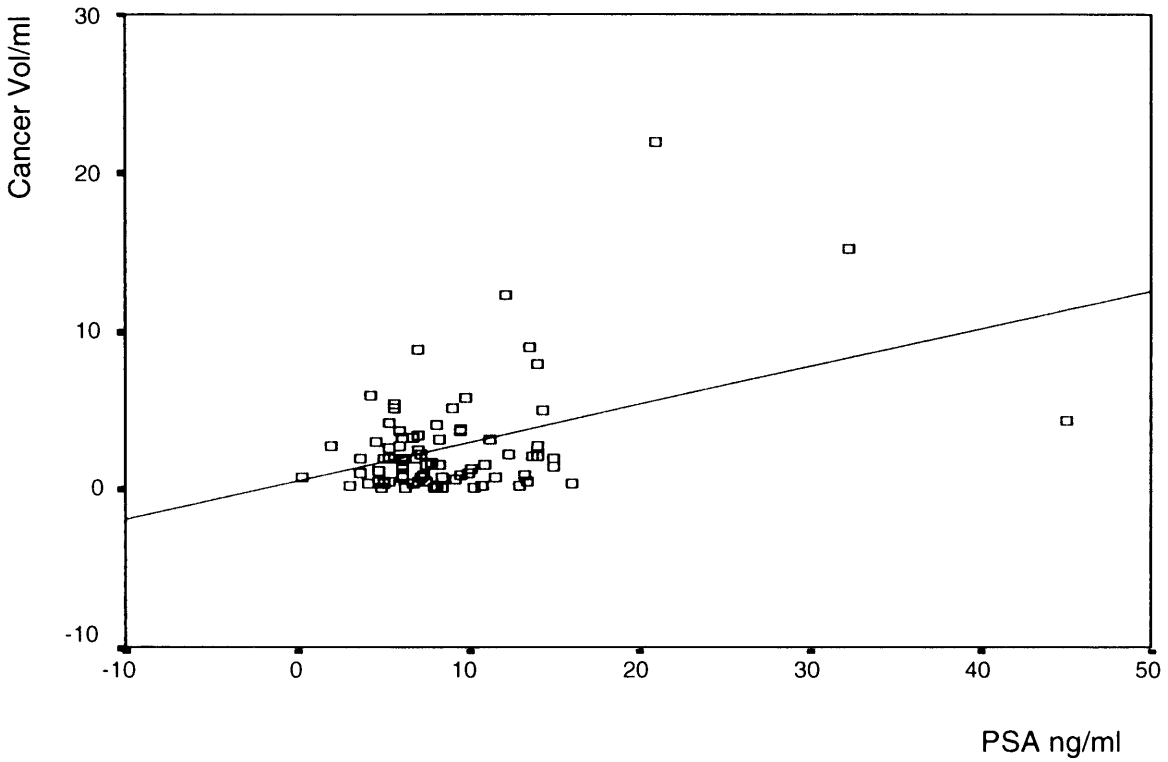

Fig 28: Relationship between serum PSA and volume of prostate cancer.

The volume of cancer was calculated from the scanned images; the area of the cancer was measured from the scanned images and multiplied by the thickness of the slices. The volume so calculated for each specimen was multiplied by a correction factor for shrinkage of 1.22. The volume of prostate cancer was plotted against the serum PSA. There is a correlation between the serum PSA and the volume of prostate cancer, spearman rho = 0.36, this is highly significant with a p value of 0.01).

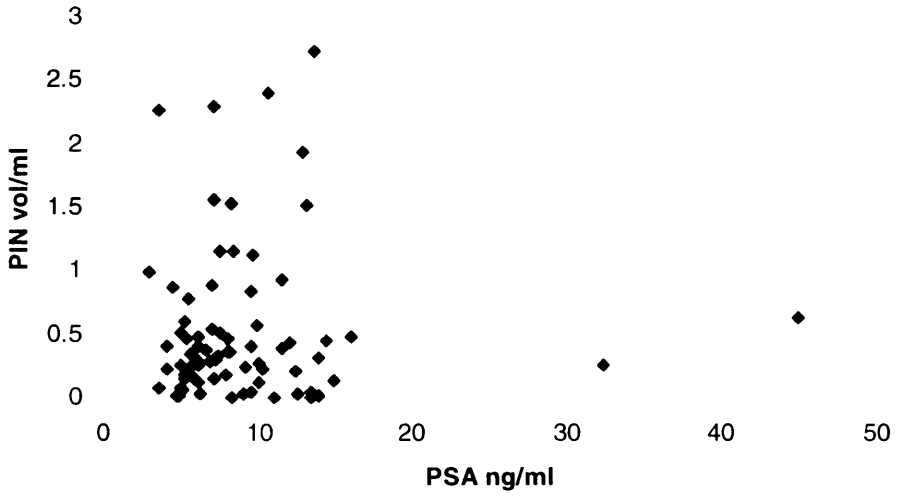

Fig 29: Relationship between serum PSA and volume of prostatic intraepithelial neoplasia.

The volume of PIN was calculated from the scanned images using Kontron KS400 software to determine the area of the regions of interest. This was multiplied by the slice thickness (3.46mm) and the shrinkage factor (1.22). The volume of PIN was plotted against serum PSA (in ng/ml). There was no correlation between the volume of PIN and serum PSA. Therefore, PIN using the model developed in this piece of work does not appear to contribute significantly to PSA.

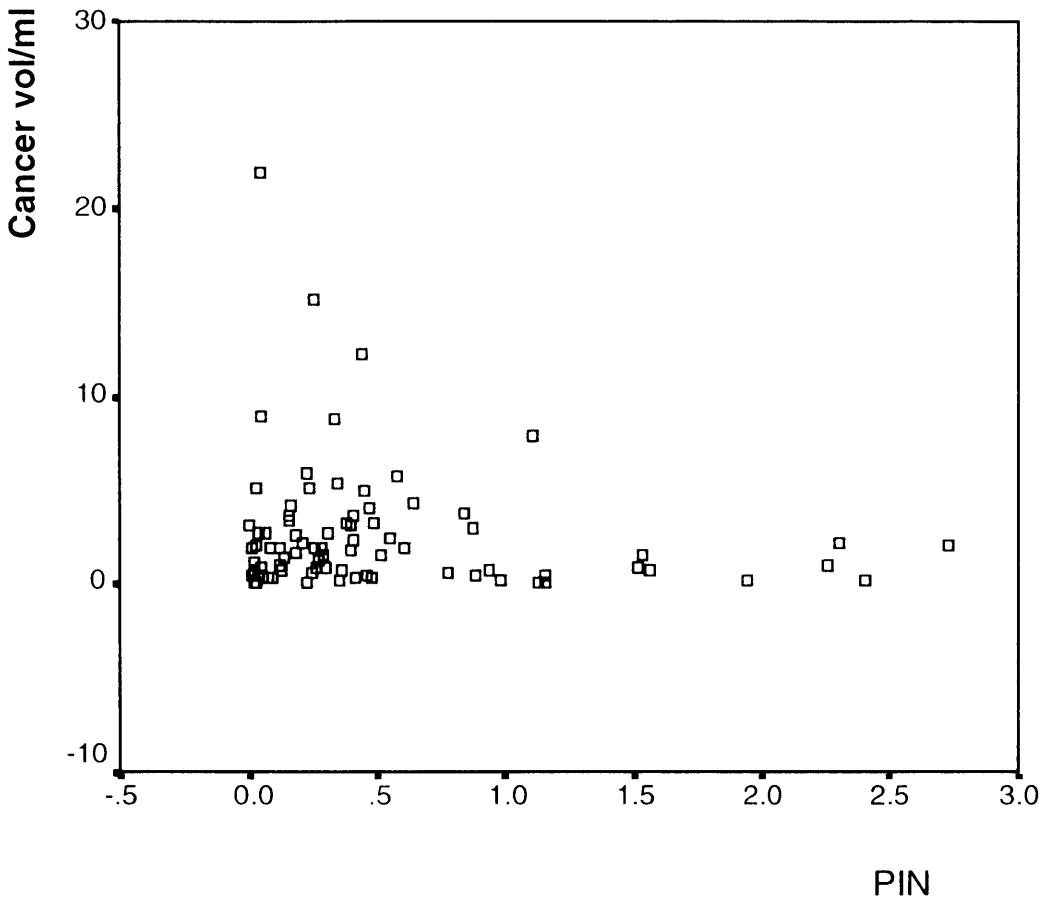

Fig 30: Relationship between PIN volume and cancer volume.

The volume of PIN was compared to the volume of cancer. Some authours have suggested there is a strong and significant inverse relationship between the volume of PIN and cancer. Hypothesising that this may be further evidence to support the view that PIN develops into prostate cancer[99]. A weak inverse relationship was detected between the volume of PIN and prostate cancer (correlation coefficient = -0.155), but this does not reach significance (p = 0.183). Therefore this data does not support the work of de la Torre et al [99].

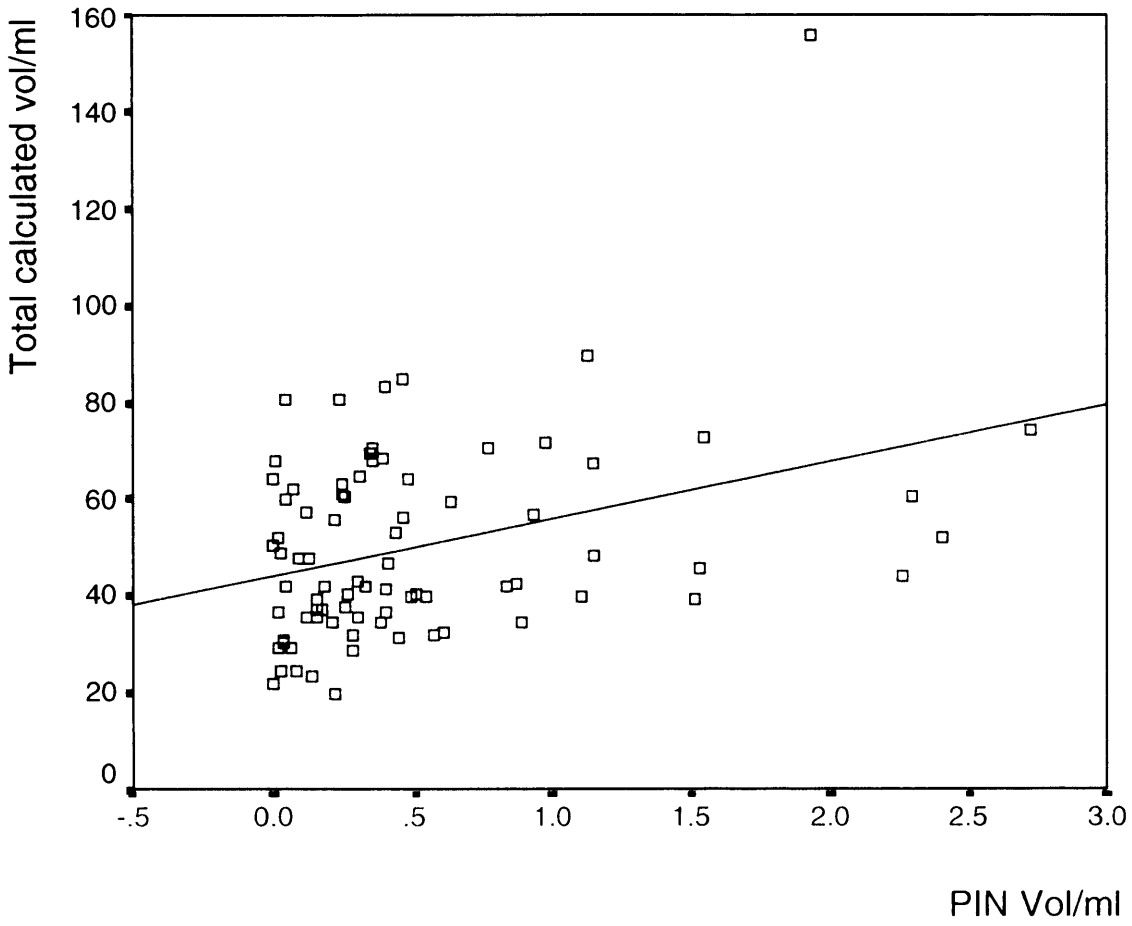

Fig 31: Relationship between PIN volume versus total calculated volume.

The total volume of the gland was calculated using the prostate maps, and this was compared with the volume of PIN. There was a significant correlation with a coefficient of 0.4(p=0.03). This may imply that both conditions respond to common stimuli, e.g. testosterone.

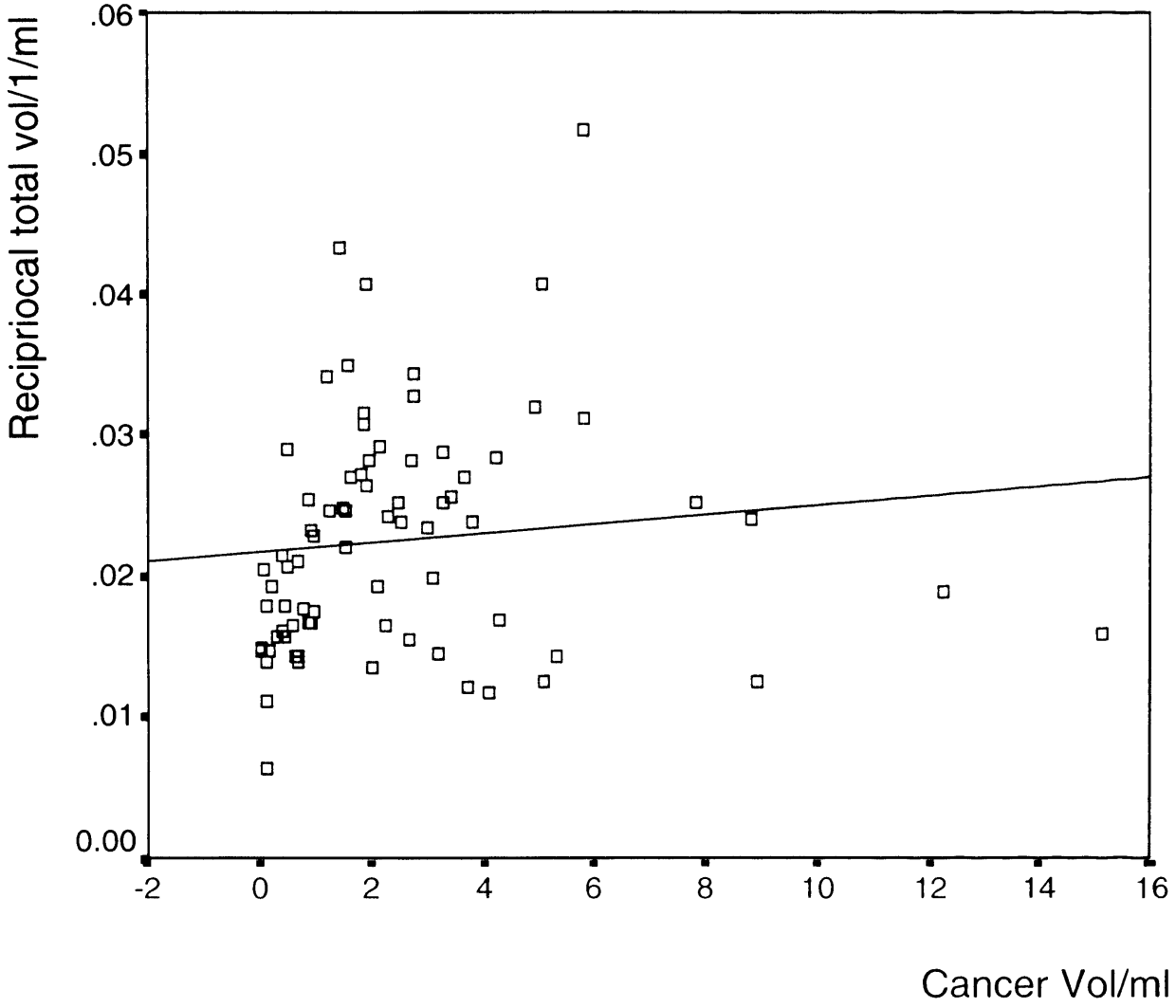

Fig32: Cancer volume versus reciprocal of total volume.

The volume of cancer was compared to the volume of the gland measured from the templates. The volume of cancer is inversely related to the total volume of the gland with a correlation coefficient of 0.3, and a p value of 0.01.

This may be due to the fact that in the majority of patients in this data set, the trigger for a biopsy is PSA, and if the PSA reaches a critical threshold then the patient is offered a biopsy. Since both benign and malignant tissue can produce PSA, therefore a patient may be offered a biopsy if he has a large benign gland with minimal tumour - the majority of the PSA being generated by benign tissue; or a small gland with a high volume of tumour - the rise in serum PSA being due to prostate cancer.

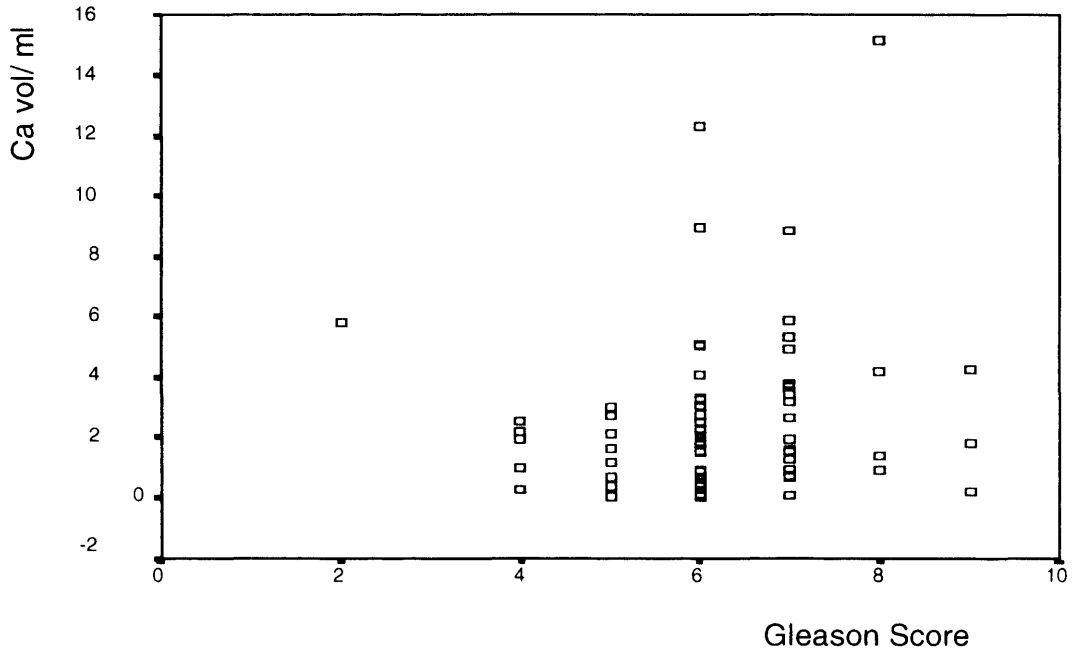

Fig 33: Cancer volume versus Gleason score.

The Gleason score is a predictor of outcome in patients with prostate cancer[131;250]. Tumour volume correlates with stage and is also a prognostic factor. Therefore there may be a relationship between Gleason score and cancer volume. We investigated this. No relationship could be demonstrated between tumour volume and Gleason score.

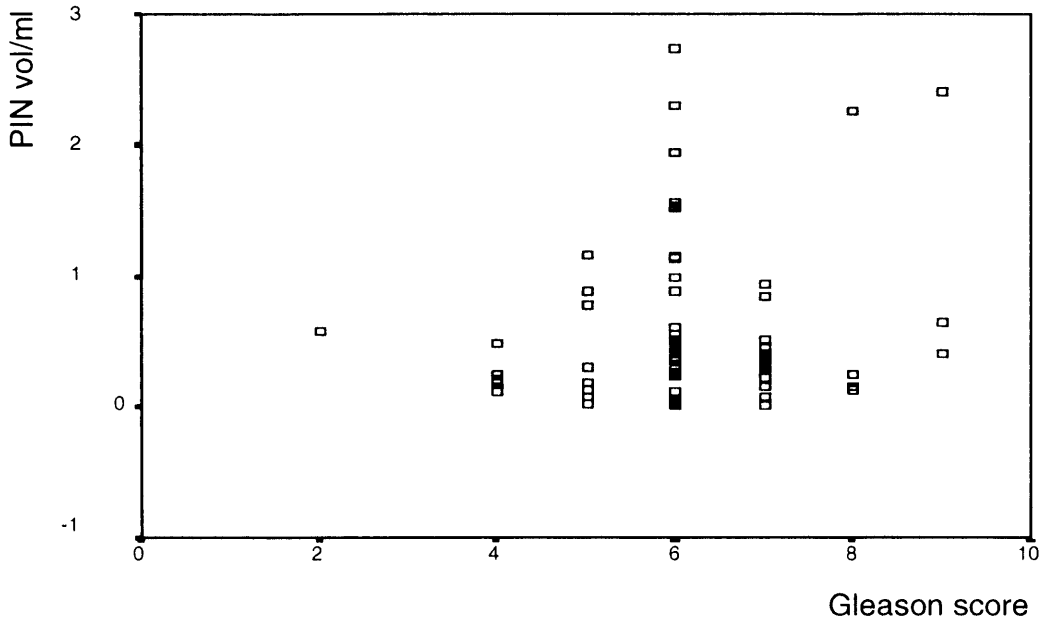

Fig 34: PIN volume versus Gleason score.

The volume of PIN was calculated using the templates, and this was compared to the Gleason score of the prostate specimens. There is no correlation between these two variables.

6.2: Results 2:
Immuno-histochemical determination of p_{53}, bcl_2, bax-alpha, and PSA in benign prostate tissue, PIN, and prostate cancer.

Areas of cancer, and PIN were identified on H&E sections of microscope slides obtained from radical prostatectomy specimens from 92 hormone naïve patients, and areas of cancer were identified from radical prostatectomy specimens obtained from 14 patients treated with three months of androgen ablation. It is difficult to identify areas of PIN after androgen ablation. Low power views were used to identify the areas of interest.

The mean age of the patients was 64.1 (range 46-71) years, mean PSA 9.6 (range 1.9-32.40) ng/ml, 77 patients had organ and capsule confined disease, and 29 patients had extracapsular disease. The mean Gleason score was 6.1 (range 2 to 9). All patients were fully staged and were NOM0.

Specimens obtained from 19 patients who had a TURP for benign disease were used as controls

For p_{53} the D07 antibody was used, and nuclear positivity was sought, the presence of positive staining was regarded as positive. The presence of p_{53} was sufficient for a specimen to be labelled as positive regardless of the percent of

cells being stained positive [312;421]. In addition the intensity of the staining was compared (Table 25). There was no significant difference in the intensity of staining between the groups studied.

For Ki-67 staining, the MIB-1 antibody was used, in order to obtain a proliferation index. 1000 cells were examined at high power and the proliferation index was the percentage of cells staining tumours positively with MIB-1. Brown granular nuclear staining was considered positive for Ki-67.

For bcl_2 and bax cytoplasmic staining was used to determine a positive result. For bcl_2 the Dako M887 antibody was used, and for bax the Santa Cruz bax N20 antibody was used. The presence of positive staining cytoplasmic cells was regarded as positive; in addition for bcl_2 the proportion of positive staining cells in the region of interest was noted. The intensity was also measured for both of the antibodies.

The crude data is available in Appendix 11.

Table 24

Percentage of P53 positive cells per slide.

	N	Minimum	Maximum	Mean	Std. Deviation
Benign	19	0	0	.00	.000
PIN	91	0	100	3.46	15.859
Ca	92	0	100	6.96	23.334
LHRH Treated	12	0	100	25.83	39.648

Table 25

Intensity of Staining of P53 Staining.

	N	Minimum	Maximum	Mean	Std. Deviation
Benign	19	0	0	.00	.000
PIN	92	0	3	.10	.446
CA	92	0	2	.14	.459
LHRH	14	0	3	.43	.852

Table 26

Percentage of Bcl2 positive staining cells

	N	Minimum	Maximum	Mean	Std. Deviation
Benign	19	0	0	.00	.000
PIN	92	0	100	47.93	36.736
Ca	92	0	100	15.98	29.800
LHRH	14	0	100	55.71	46.029

Table 27

Intensity of Staining of BCL-2 Staining.

	N	Minimum	Maximum	Mean	Std. Deviation
Benign	19	0	0	.00	.000
PIN	92	0	5	1.39	.889
CA	92	0	2	.35	.563
LHRH	14	0	3	1.00	.961

Table 28

Percentage of Ki 67 positive staining cells

	N	Minimum	Maximum	Mean	Std. Deviation
Benign	19	0	0	.00	.000
PIN	92	0	40	4.50	6.010
CA	92	0	35	5.68	6.304
LHRH	14	1	25	2.79	6.399

Table 29

Intensity of Staining of Bax Staining.

	N	Minimum	Maximum	Mean	Std. Deviation
Benign	19	0	2	1.38	.650
PIN	84	0	3	1.80	.741
CA	84	0	3	1.88	.735
LHRH	14	.00	2.00	1.40	.986

Table 30

Intensity of Staining of PSA Staining.

	N	Minimum	Maximum	Mean	Std. Deviation
Benign	19	3	3	3.00	.000
PIN	92	1	3	2.59	.577
CA	92	0	2	.14	.459

		Total Number	No. +	Mean no of +ve cells +/- SD	Staining intensity +/- SD
P53	**BPH**	19	0	0	0
	PIN	92	5	3.46 +/- 15.86	0.1 +/- 0.45
	CaP	92	9	6.96 +/- 23.33	0.14 +/- 0.46
	LHRH	14	4	25.83 +/-39.65	0.43 +/- 0.85
Bcl2	**BPH**	19	0	0	0
	PIN	92	75	47.93 +/- 36.74	1.39 +/- 0.89
	CaP	92	27	15.98 +/- 29.8	0.35 +/- 0.56
	LHRH	14	9	55.71 +/- 46.03	1+/- 0.96
Baxalpha	**BPH**	13	13	100	1.38 +/- 0.65
	PIN	84	78	100	1.8 +/- 0.74
	CaP	84	80	100	1.88 +/- 0.74
	LHRH	10	10	100	1.4+/-0.96
Ki-67	**BPH**	19	0	0	
	PIN	92	91	4.5 +/- 6.01	
	CaP	92	92	5.68+/- 6.3	
	LHRH	14	14	2.79 +/- 6.4	
PSA	**BPH**	19	19	100	3 +/- 0
	PIN	92	92	100	2.6 +/- 0.56
	CaP	92	92	100	2.15 +/-0.76

Table 31: Summary of results obtained by immunohistochemisty

	Number of + specimens					Number of cells			Staining intensity			
Comparisons	**P_{53}**	**Bcl 2**	**Bax**	**Ki-67**	**PSA**	**P_{53}**	**Bcl2**	**Ki-67**	**P_{53}**	**Bcl2**	**Bax alpha**	**PSA**
PIN and BPH	NS	<.001	NS	<.001	NS	0.71	<0.001	<0.001	0.71	<0.001	0.06	**0.0116**
CaP and PIN	NS	**<.001**	NS	NS	NS	0.61	**<0.001**	0.16	0.62	**<0.001**	0.57	0.0002
CaP and BPH	NS	<.006	NS	<.001	NS	0.51	0.04	<0.001	0.51	0.04	0.03	**<0.001**
LHRH and CaP	.07	**0.015**	NS	NS	NS	0.19	0.006	**0.006**	0.27	0.016	**0.04**	

Table 32: Comparison of the benign, PIN, and cancer using the Mann Whitney U Test and the difference of the means for the number of cells and staining intensity. P-values in bold denote negative differences, i.e. the second factor is higher than the first. Otherwise the first factor is higher.

Chapter 7: Discussion

The volume of the prostate glands was calculated using digital images of the slices, which were corrected for the shrinkage factor. This calculated volume was compared to the weight of the gland. There was good correlation (correlation coefficient of 0.967). This would support this technique as a valid method of calculating prostate volumes.

7.1 Relationship between total volume of gland, volume of PIN, benign volume of gland, and volume of cancer.

Tumour volume as assessed in total prostatectomy specimen correlates with prognosis, especially tumour volumes greater than 4.0cm^3 even in patients who have relapsed [93;367]. When comparing the tumour volume as a marker for recurrence, some have suggested that it is a better predictor of recurrence after radical prostatectomy than either Gleason score or preoperative PSA[293].

Tumour differentiation decreases and DNA patterns deteriorate as the tumour volume increases. Some groups have shown that tumour volume correlates with histological grade[301;365] and although it did predict progression, in a multivariate analysis it was not an independent predictor of recurrence [301]. In

our series tumour volume did not correlate with Gleason grade.

In this study the volume of cancer was related to the stage ($p<0.001$). The cancer volume also had an inverse correlation with the benign and the total volume of the gland ($p<.001$). This may be due to the fact that to trigger a biopsy and so the possible diagnosis of prostate cancer the patient needs to reach a threshold PSA. PSA can either be produced by the benign tissue, i.e. a patient will have a large gland with a small volume of cancer; or by malignant tissue and so a patient will have a small gland with a large volume of cancer sufficient to produce the PSA which leads to serum levels that trigger a biopsy.

Additionally this work has demonstrated that the size of the gland correlates with the volume of PIN. Both BPH and PIN increase with age and require androgens. Therefore, the stimulus for these lesions is probably identical, with PIN developing in the peripheral zone and BPH developing in the transition zone. If we accept that PIN is a premalignant lesion, then large glands have a corresponding increased volume of PIN, which would predispose to cancer. Therefore gland volume is a predictive factor for prostate cancer. The European Randomised Study of Screening for Prostate Cancer (ERSPC) screened a total of 8,621 men. Using a logistic regression model this group determined the most important predictors for prostate cancer was PSA, followed by prostate volume, DRE, and TRUS findings, in that order[205]. Thus

a large gland irrespective of the PSA may be indicative of prostate cancer, due to the increased volume of PIN.

No significant correlation was detected between the volume of PIN and the volume of cancer in this study. This is in contrast to other studies. De la Torre et al did demonstrate an inverse relationship between volume of PIN and the volume of cancer. Implying that as PIN transforms into prostate cancer, the volume of PIN decreases as the volume of cancer increases, however they did not differentiate between high and low grade PIN [99]. Alexander et al demonstrated a significant correlation between PIN volume and the weight of the gland and the cancer volume [6].

7.2 Relationship between volume of PIN, volume of benign tissue, volume of cancer and serum PSA.

In this study it has been shown using a regression model that the serum PSA significantly correlates with the volume of the gland and the volume of prostate cancer. In a multivariate analysis this correlation appears to be independent. Using SPSS ANOVA software, we have calculated one gm of benign prostate tissue correlates with 0.07ng/ml of serum PSA, and one gm of malignant tissue to 0.8 ng/ml of serum PSA.

Stamey et al in their seminal paper calculated that the serum PSA was related

to tumour volume, and that prostate cancer produced more PSA than benign tissue [366]. In the cohort of patients with prostate cancer no significant correlation was calculated between the volume of benign tissue and the serum PSA. In seven separate patients undergoing a Millen's retro pubic prostatectomy for benign disease they calculated that on average one gram of benign tissue corresponded to 0.29 ng/ml of PSA in the serum[366]. This group further studied the relationship between cancer and serum PSA and concluded that prostate cancer elevates serum PSA twelve fold compared to BPH and so one gram of cancer increases serum PSA by 3.5ng/ml[365]. However, of the 102 radical prostatectomy specimens used to calculate this figure only 48 were completely confined to the prostate, and therefore the total tumour volume was only measured with any degree of accuracy in less than half of this cohort. Stamey also detected a correlation between serum PSA and prostate weight, but not with the volume of benign tissue[365]. Partin et al studied the relationship between tumour volume, benign gland volume and serum PSA in 350 radical prostatectomy specimens and 72 men with benign prostatic hyperplasia. A correlation was found between the cancer volume and PSA, no correlation was detected between serum PSA and the volume of benign tissue[300]. Later, the same group in a series of 47 radical prostatectomy specimens detected a significant correlation between serum PSA and the weight of the gland and volume of malignant tissue [412]. This has been confirmed in this study and by others[6]. Ronnett et al demonstrated that in small volume tumours i.e. 0.5cc or

less, the weight of the gland correlates with the PSA, and the tumour volume does not [326].

In a series of 159 cases with a clinically benign diagnosis and using TRUS to calculate the volume of the gland, and of the transition zone. Aarnich et al calculated that one-gram of the transition zone contributed to 0.150ng/ml of serum PSA and the remainder of the prostate contributed 0.077ng/ml per gram. The mean contribution for one gram of prostate was 0.092ng/ml [2]. An earlier study of 472 men calculated a value of 0.72 ng/ml per gram of benign prostate tissue[86]. The relationship between prostate volume and free PSA has also been studied. Using a multiple linear regression analysis in a model containing both free and total PSA values obtained from 681 men, free PSA was the only significant predictive variable of prostate volume[271]. Studies have examined not only the effect of prostate volume on serum PSA but also age, it has been shown that serum PSA increases with age[86;102;325].

In this study we have demonstrated that there is no relationship between the volume of PIN and serum PSA.

Brawer postulated that for PSA to enter the serum it must traverse the basal cell layer, epithelial basement membrane, stroma, capillary basement membrane and the endothelial cells. In PIN there maybe disruption in the basal

cell layer and so it was postulated that serum PSA might be increased in patients with PIN[50]. In a series of 81 patients, treated for bladder outlet obstruction by retro pubic Millen's prostatectomy and TURP, which included 25 men with PIN and 26 with BPH. The median serum PSA levels were significantly higher in the group with PIN (4.0 ng/ml) compared to the men with BPH (2.1ng/ml)[53]. Using needle biopsies Morote et al identified 321 men with benign prostatic hyperplasia, 17 had PIN present and 304 had no PIN, there was a small but insignificantly higher total PSA in patients with benign tissue and associated PIN than those with no PIN (7.7ng/ml vs. 7.2ng/ml, $p>0.05$)[270]. Fowler et al carried out TRUS guided biopsies on 1961 consecutive men with suspected prostate cancer as part of a screening protocol. 93 patients had PIN diagnosed in the absence of cancer, and 957 patients had a benign diagnosis without PIN. The mean total PSA was 5.6 and 4.6 ng/ml, and the percentage free was 20.6 and 21.5 respectively. The difference in the percentage of free PSA was not significant, but the total PSA was significantly higher in the group with PIN ($p=0.02$)[120]. Bostwick et al also detected an increased PSA in patients with PIN diagnosed on needle biopsy compared to a benign diagnosis[48]

A study of 15 patients with BPH, 46 with PIN, and 16 with localised prostate cancer, the total mean PSA was significantly different between the three groups i.e. 2.78ng/ml for BPH, 4.89ng/ml for low grade PIN, 8.21ng/ml for

high grade PIN, and 12.70ng/ml for cancer. The mean free to total ratio was lower in patients with low grade PIN (25%) or high grade PIN (21%) compared to cancer (14%) but this did not reach significance[198]. Minardi et al carried out a retrospective study of 87 patients, 30 patients with BPH, and 24 with high grade PIN, 8 with low grade PIN and 25 with carcinoma of the prostate[258]. The mean free to total ratio was 24.2% in those with BPH, 17.6% in the high grade PIN, and 13% in the prostate cancer group. These differences were significant. The mean values for the total PSA i.e. 6.17 ng/ml, 6.89 ng/ml, and 8.02 g/ml respectively, were not statistically significant[258]. Ramos et al similarly followed up 48 men who were diagnosed with PIN without prostate cancer. This cohort was compared to a group diagnosed with prostate cancer and a group with benign prostatic hyperplasia in the absence of PIN. The percentage free PSA was significantly lower in patients with prostate cancer, but there was no difference in the percentage free PSA in the men with PIN and those with BPH (20.8+/- 7.1% and 20.1+/-7.3%, respectively) [316]. In a study of 81 consecutive patients undergoing radical prostatectomy, Morote et al found no significant difference in the total serum PSA between the specimens that contained PIN and cancer compared to those with cancer alone. But the free to total serum PSA was significantly lower in the group with PIN, but in a multivariate analysis PIN did not contribute to free serum PSA [269]. In the large Austrian screening study of 1474 patients, the mean total PSA of patients with BPH, PIN and cancer was 6.0, 5.9, and 8.7ng/ml, with the

percentage free ratio being 21.9, 15.0 and 12.1% respectively. With regard to total PSA there was no significant difference between BPH and PIN, but with regard to the percentage free PSA there was a significant difference between the BPH group and all the other groups, and the PIN and cancer groups[170]. This study could not identify a clear cut off between the groups due to a significant overlap[170].

Alexander et al have shown that PIN volume does correlate with serum PSA concentration[6]. This group studied 194 whole mounted radical prostatectomy specimens and used a grid counting technique to determine the volume of PIN, and prostate cancer. They, however, concluded that the relationship between serum PSA and PIN was an artefact, in a multiple regression analysis adjusting for cancer volume, gland weight, Gleason score, and extraprostatic extension, log PIN volume did not contribute to log serum PSA [6].

Ronnet et al using 65 whole mount radical prostatectomy specimens weighing less than 65 gm and with small tumour volumes of <0.5mls, found no correlation between PSA and tumour and PIN volume, but did find a correlation between serum PSA and total gland weight[326].

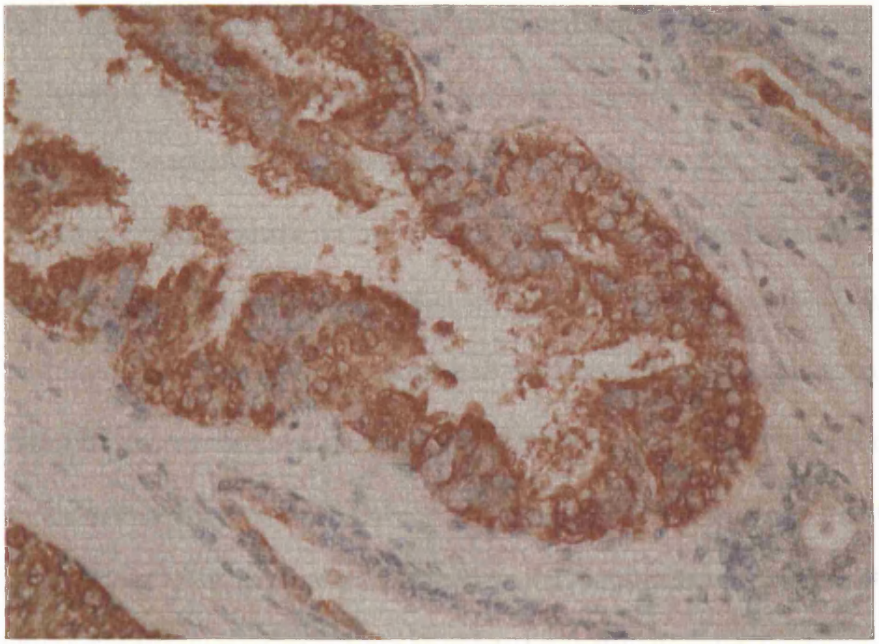

Fig35: PIN stained with PSA x 40

Fig36: Cancer stained with PSA x 40

7.3 P_{53} in benign, PIN and malignant prostate tissue.

Expression of p_{53} increased in a stepwise manner from benign to intra epithelial neoplasia to malignancy. 0/19 benign, 5/92 PIN, and 9/92 cancer specimens were positive for p_{53}. This increase was not significant. Loss of p_{53} was thought to be a common event in localised prostate cancer[274], however more recent studies have not supported this view[273]. In only two out of the five PIN specimens expressing p_{53} was there also concurrent expression of p_{53} in the cancer specimens implying aberrant expression of p_{53} is not required for progression. Furthermore sequencing studies of aberrant p_{53} in both PIN and cancer in the same specimen have revealed that the sites of the point mutations are not identical and therefore PIN and prostate cancer may arise from a different clone with in the same specimen[377;426]. In addition we have shown that progression to cancer from PIN can occur in spite of the loss of aberrant p_{53}. Loss of p_{53} is probably a late change in prostate cancer[369], and not related to the early genesis of this disease.

Androgen ablation led to proportionally increased expression of p_{53}; this however did not reach significance (p=0.07). There may be a number of explanations for the increase in p_{53}. Androgen ablation leads to the withdrawal of a necessary stimulus for the prostate, the cells of the gland involutes, and a p_{53} dependant pathway may cause this. In addition increased bcl_2 inhibits the nuclear translocation of p_{53}[21] and so may increase the amount

of free wild type p_{53} antigenically available for the immunohistochemistry antibody to react with.

Increased p_{53} after androgen ablation may also be a predictor of relapse. An analysis of 39 patients, who had received neoadjuvant LHRH treatment prior to definitive surgery, demonstrated 21 patients that were positive for p_{53}. 90% (19 patients) of these patients relapsed. Therefore the increased expression of p_{53} associated with androgen ablation may predict relapse [312].

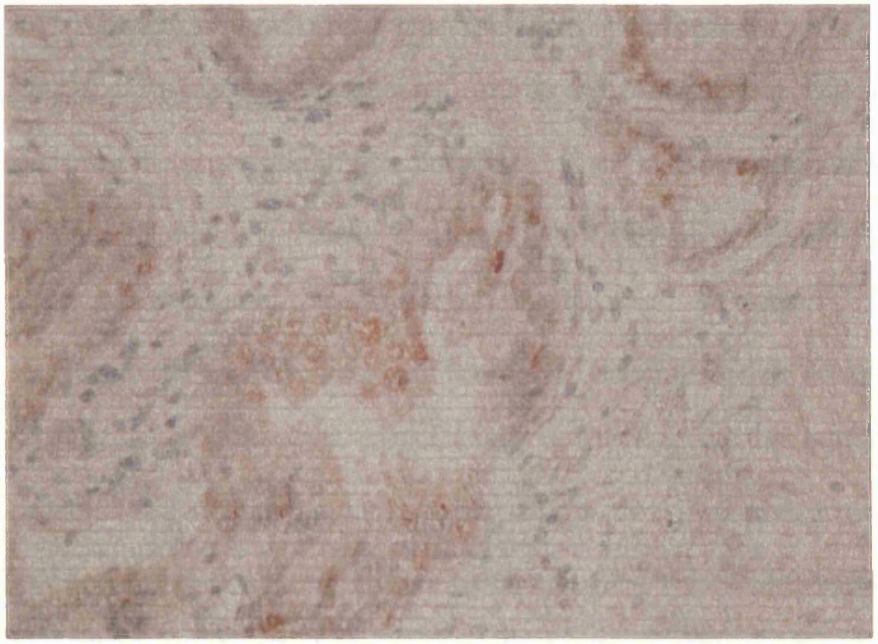

Fig37: PIN stained with P_{53} x 40

Fig38: Cancer stained with P_{53} x 40

7.4 Bcl_2 in benign, PIN and malignant prostate tissue.

Bcl_2 is always present in the stem cells of the prostate; therefore we only measured the expression of bcl_2 the luminal cells. The expression of bcl_2 was significantly different between the three groups studied. The expression of bcl_2 in the prostate cancer was comparable with the results of others[20;74;238;239]. It was greatest in the PIN slides, and least in the benign tissue. In 22 specimens bcl_2 staining was detected concomitantly in PIN and cancer. Bcl_2 infers a degree of immortality and resistance to the cells from cytotoxic treatment, if PIN is a pre malignant lesion and not an epi phenomenon, this would imply that PIN luminal cells have a survival advantage. Others also share this view[41;187].

Expression of bcl_2 allows cells to remain viable despite androgen ablation [22]. In this study androgen ablation led to an up regulation of bcl_2 and therefore it may be postulated that androgen ablation selects out clones with a survival advantage[246]. Others have also supported this finding[394]. Up regulation of bcl_2 may also play a role in the development of radio resistant clones[328] and increased expression of VEGF resulting in higher microvessel density [116].

An interrelationship exists between p_{53} and bcl_2. Up regulation of bcl_2 can inhibit the action of the pro apoptotic p_{53}[21] and conversely p_{53} can up regulate bax and down regulates bcl_2[261].

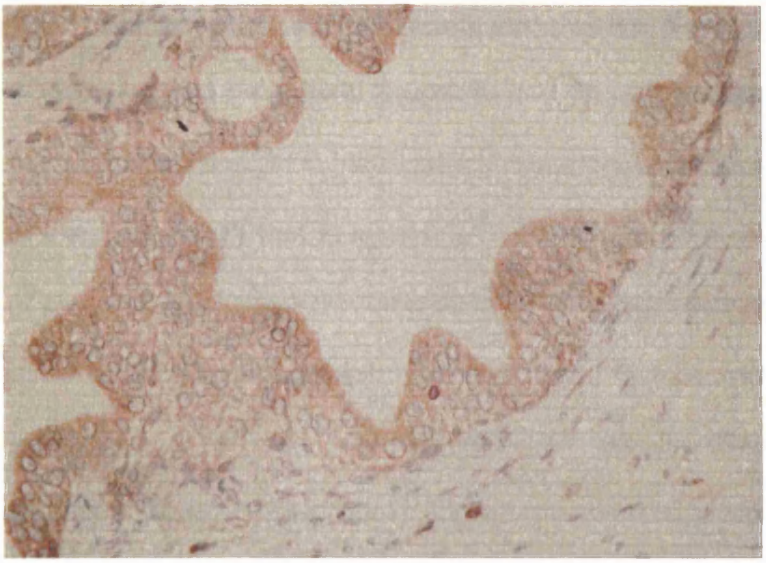

Fig 39: Bcl_2 positive staining PIN x 40.

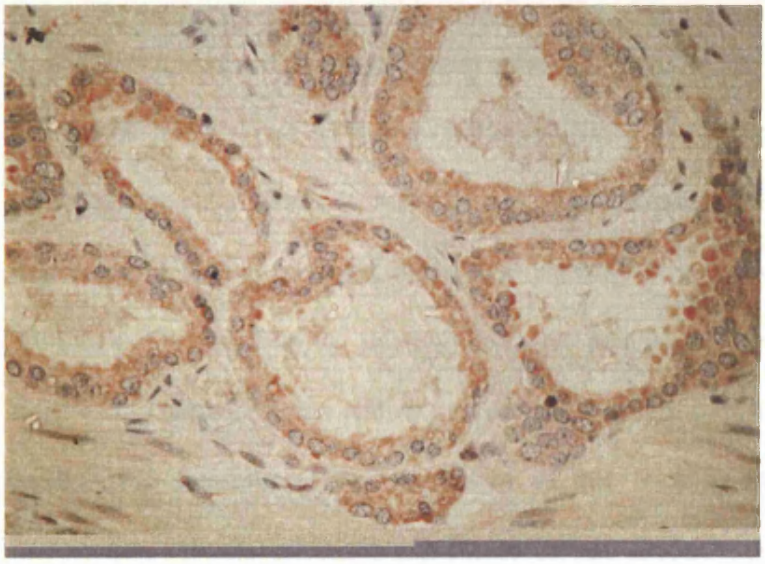

Fig 40: Bcl_2 positive staining cancer x 20.

7.5 Bax in benign, PIN and malignant prostate tissue.

A predetermined set point is thought to exist in cells for the ratio of bcl_2 and bax, and this is thought to play a critical role in the response of malignant cells to therapies, which lead to apoptosis[292]. Bax inactivation abolishes induction of apoptosis by chemopreventitive drugs[178]. The expression of bax was almost uniform in all the groups. However, the staining intensity increased significantly from BPH to cancer and there was inactivation of bax with androgen ablation. This would imply up regulation of bax from benign to malignant tissue, so cancer cells are selectively more susceptible to chemotherapeutic/hormonal intervention. When comparing the specimens, which have been treated with androgen ablation there is a decrease in staining intensity, this may be because the susceptible cells have undergone apoptosis and only the resistant clones are left. The ratio of bax to bcl_2 is important and in the group treated with androgen ablation there is an increase in the expression of bcl_2, and so further adding weight to the argument that in this group androgen ablation is selecting resistant clones. Bax inactivation is important in human carcinogenesis, and contributes to tumour progression. Although the number of positive staining specimens does not show a significant change, but the staining intensity does, and therefore a quantitative method for determining the expression of bax is required to confirm its importance. If one measures expression of bax exclusively in terms of the number of specimens staining positive, then its loss does not appear to be

important in the initiation of prostate cancer. Others share this view[187; 204].

Fig41: Bax positive staining PIN x 40.

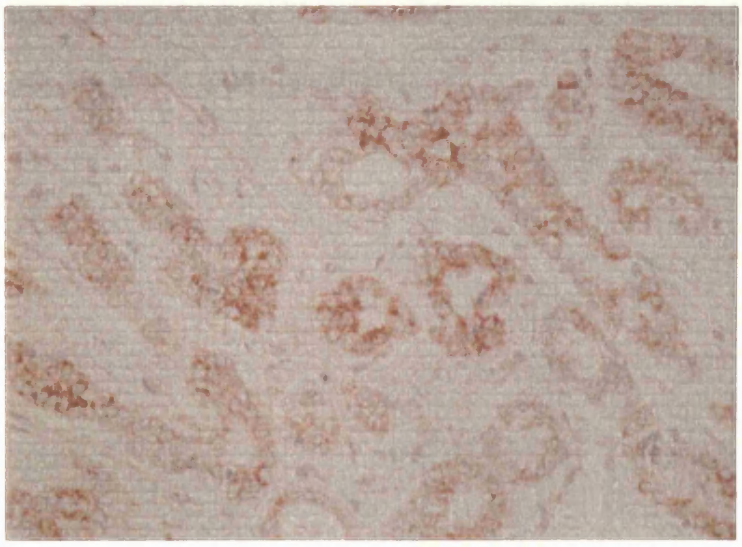

Fig 42: Bax positive staining cancer x 40.

7.6 Ki-67 in benign, PIN and malignant prostate tissue.

In this study there is a significant increase in the expression of Ki-67 between benign tissue, and PIN or cancer. However, the difference between PIN and cancer is not significant. Others have also detected low expression of Ki-67 in benign tissue [285]. Ki-67 positive cells shift from basal to luminal as one moves from benign, to PIN and cancer. This would imply, that not only is there a decrease in the level of apoptosis in cancer, but there is also increased cell production from benign to PIN or cancer and this is localised to the areas of malignant tissue.

The prostate is a hormone responsive organ and cell production is decreased by androgen ablation. Therefore this explains the decrease in Ki-67 expression associated with androgen ablation.

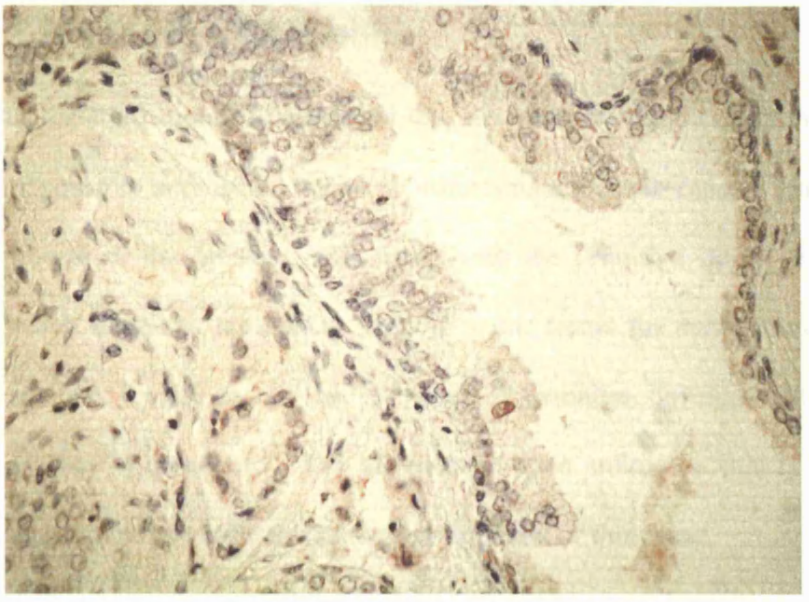

Fig 43: Ki-67 positive staining PIN x 40.

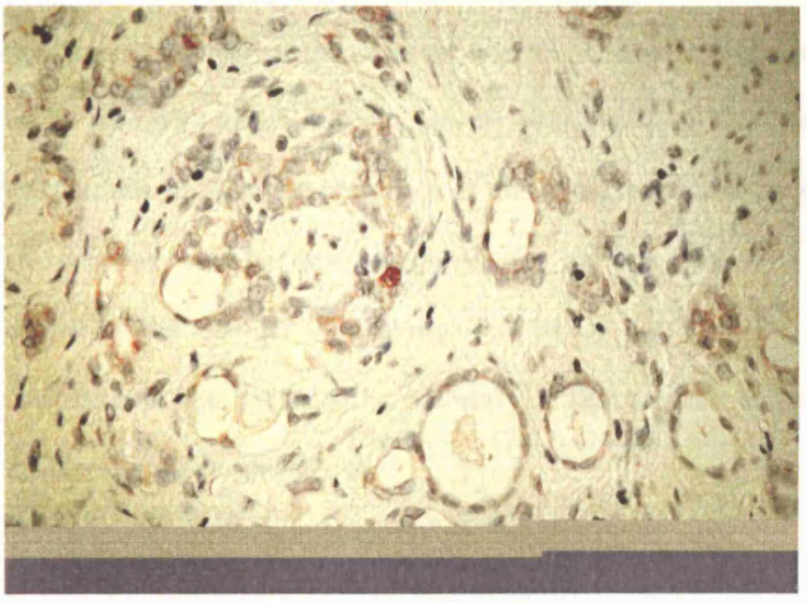

Fig 44: Ki-67 positive staining cancer x 40.

Chapter 8: Conclusion.

In the third month of gestation the mesenchyme surrounding the urethra, in response to androgens, begins to differentiate into the capsule and the smooth muscle of the prostate. This along with the primitive lining of the urethra, which develops into ducts and the acini, forms the basic structures of the prostate. By the end of the 15th week of gestation, development of the basic prostate is complete. The dependence upon androgens that is so necessary early on will be hallmark of the development of this gland.

The prostate in response to androgens grows through out adult life. This results in a number of pathological conditions, both benign and malignant, which are major health issues. Yet, any intervention on the prostate gland is fraught, due to the close proximity of this gland to the urinary sphincter and the neurovascular bundles responsible for potency. Lowsley traditionally described the prostate as having five lobes, but a more useful zonal classification was proposed by McNeal[248]. The majority of prostate cancer develops in the peripheral zone.

Prostate cancer is the second commonest cause of cancer related death in males, yet the optimal management of localised carcinoma of the prostate represents a paradigm of uncertainity[333]. A number of risk factors have been implicated; these include age, race, family history, diet and androgens.

Prostatic Intraepithelial Neoplasia and Prostate Cancer.

In this thesis, the various options have been outlined for the management of prostate cancer. The rational for these treatment modalities, whether it be watchful waiting, active surveillance, radiotherapy- external beam, conformal external beam, brachytherapy, or radiotherapy with adjuvant androgen ablation, surgery - open retro pubic, perineal, laparoscopic (extra or transperitoneal) has been discussed. Yet, despite the mushrooming options for the management of localised carcinoma only relatively recently has evidence been available that intervention does have any positive impact[33].

The increased interest in prostate cancer, has led to the discovery of a number of lesions which were thought to be premalignant, one of these is prostatic intraepithelial neoplasia[46], a lesion which exhibits the cytological changes of prostate cancer, but the basal cell layer is retained. Prostatic intra epithelial neoplasia has to be distinguished from atypia, transitional cell metaplasia, basal cell hyperplasia, clear cell cribriform hyperplasia, normal ejaculatory ducts, and seminal vesicle epithelium, high grade transitional cell carcinoma involving the ducts and acini, cribriform acinar and cribriform ductal prostatic carcinoma.

Initially prostatic intra epithelial neoplasia was thought to exist in three distinct forms, but this has been distilled to two, and then only one type, high-

grade prostatic intraepithelial neoplasia. Studies have shown that this lesion may precede prostate cancer, and can be detected in men as early as their third decade. The incidence of PIN in screening studies is between 0.7% and 16.5%, in TURP specemens it is between 2.8 and 3.2%. The discrepancy is probably due to the TRUS biopsies sampling the peripheral zone, and TURP chips being obtained from the transition zone.

PSA is a 240 amino acid glycoprotein serine protease with a molecular weight of 33,000. Initially used in forensic medicine it has evolved into one most important tumour markers used in medicine. The relationship between PIN and serum PSA had not been clearly defined, and it is hoped that this work has helped to elucidate this better.

To investigate the relationship between PIN, prostate cancer and serum PSA, this prospective study was undertaken. Prostate maps of fresh specimens obtained from radical prostatectomy specimens were constructed. The prostate was initially placed in 10% formaldehyde for 72 hours. The specimen was then sliced. The slices were scanned, and then fixed. Slides were prepared of the slices and areas of tumour and PIN identified and transcribed onto the prostate maps. The volume of cancer, PIN and of the total specimen was calculated from the scanned images, by measuring the area of interest from the scanned images and then multiplying it by the thickness of the slices. The volume so

calculated for each specimen was multiplied by a correction factor for shrinkage. The resulting volumes were compared to the serum PSA.

The volume of PIN is proportional to the total volume of the gland and the volume of benign tissue with in the gland. The volume of cancer is inversely proportional to the benign and total volume of the gland, and directly related to the stage of the cancer.

PSA increases with the volume of benign and malignant tissue. One gram of benign tissue correlates with 0.07 ng/ml of serum PSA, and one gram of malignant tissue correlates with 0.8 ng/ml of serum PSA. Serum PSA does not correlate with the volume of PIN. The intensity of PSA staining decreases from benign, to PIN, to malignant tissue in a stepwise manner.

The importance of PIN is due to its predictive value for prostate cancer in up to 50% of patients with an initial diagnosis of PIN on biopsy. The relationship between PIN and prostate cancer was investigated at a molecular level using p_{53}, bcl-2, bax_{alpha} and Ki-67.

P_{53} protein is the product of a gene located on the short arm of chromosome 17. The product of the p_{53} gene has a molecular weight of 53 Kda, and the wild type p_{53} by binding to DNA as a transcription factor inhibits the advancement

of cells from the G1 to the S phase, and so initiates apoptosis in defective cells - the Guardian of the genome. A mutant p_{53} gene produces a defective protein, which can not regulate cell proliferation, and so cells may become immortal. Transfection of wild type p_{53} into immortal cell lines can lead to growth arrest and apoptosis. Lack of a functioning p_{53} confers an elevated risk of developing cancer – Li Fraumeni Syndrome[400]. The aberrant p_{53} gene product has a longer half-life than the wild type and so is detected by imunohistochemical analysis.

Initially p_{53} was thought to be an important prerequisite in localised carcinoma, and was used to predict outcome in patients at different stages of the disease with different Gleason scores. In patients who had undergone radical prostatectomy p_{53} was thought to be able to predict outcome, but the weight of evidence at present points to its role being greater in metastatic disease, and causing extra prostatic spread.

In our experiments, p_{53} expression was detected using immunohistochemical staining. The level of staining for p_{53} did not increase significantly as one progressed from benign, to PIN, and to cancer. P_{53} therefore does not appear to be important in the early genesis of prostate cancer. The presence of positive staining for p_{53} appears to be a relatively infrequent event. There are some who agree with this [421] and others who do not[356].

The discrepancy between our data and others may be due to the stage of the disease in patients. Even a recent study of 98 radical prostatectomy specimens, identified 57% of specimens were positive for p_{53}, but at a median follow up of 7.42 years (2.6 –12.9), 9 patients died from prostate cancer and 32 others progressed, implying relatively advanced disease at the time of surgery[356].

There appears to be an increase in p_{53} staining in the specimens obtained from patients who have received androgen ablation therapy prior to radical prostatectomy. However, this did not reach significance. The increase in expression may be due to androgen ablation causing apoptosis via a p_{53}-mediated pathway.

The bcl_2 gene was initially detected in B-cell follicular lymphomas. The bcl_2 proto-oncogene is located on the long arm of chromosome 18 and codes for a 26 kDa membrane protein. The bcl_2 protein inhibits apoptosis, possibly by limiting the release of pro-apoptotic enzymes from the mitochondria and so promotes survival.

Bcl_2 expression can allow prostate cancer cell lines to survive in the absence of androgens, and so can negate the therapeutic effect of androgen ablation in the management of prostate cancer[313]. Further more in animal experiments

androgen ablation led to an increase in the mRNA for bcl_2, this may support the hypothesis that trigger for the development of androgen insensitive controls is the withdrawal of androgens[246].

Bcl_2 expression in radical prostaectomy specimens is associated with higher stage and shorter progression free survival[61]. In patients being treated with radiotherapy, the expression of bcl_2 is associated with shorter time to relapse[173].

Bcl_2 expression increases from benign to PIN and then decreases in cancer. The high levels of expression of bcl_2 in PIN, would allow PIN to become relatively resistant to androgen ablation and chemotherapeutic intervention. The relative survival advantage conferred on PIN, could allow it to become the source for androgen insensitive clones. This is further supported by evidence that shows that bcl_2 expression appears to have an inverse relationship with androgen receptor expression[41].

Androgen ablation led to an up regulation in the expression of bcl_2. This allows the cells to become androgen independent[313] and also inhibits p_{53} mediated apoptosis by preventing the wild type p_{53} from translocating to the nucleus and triggering apoptosis[21]. Increased bcl2 also causes increased expression of VEGF, and so increased microvessel density, allowing cells to

survive in hostile environments[116].

Bcl associated x protein or bax is a proapototic molecule. It is a cytosolic protein which forms a heterodimer with bcl_2. This causes the bax molecule to translocate to the mitochondrial membrane and bind to the membrane bound bcl_2 protein. This binding results in inactivation of bcl_2 and cell death.

The expression of bax was ubiquitous, in benign, PIN, and prostate cancer. The staining intensity was however greater in prostate cancer ($p=0.03$) and PIN ($p=0.06$) than in benign tissue, implying that there may be increased production of bax on a cellular basis leading to increased turnover of malignant and pre-malignant cells. Androgen ablation decreased the staining of cells expressing bax and so this may lead to decreased cell turnover via the bcl_2/bax axis, especially since there is increased expression of bcl_2.

The gene coding for the Ki-67 protein is on chromosome 10. Ki-67 is a nuclear protein expressed in the G1, S, G2, and M phases of the cell cycle, but not in G0 – the resting phase of the cell cycle. Therefore MIB-1 antibody staining can be used in formalin fixed tissue to determine the proliferation rate. The alternative is thymidine incorporation assays, but for this fresh tissue is required and this is a very demanding technique. The proliferation rate as measured by Ki-67 can be used to predict extra-capsular spread[229],

recurrence[142] and outcome after surgery or radiotherapy[1]. The proliferation rate may also increase with Gleason grade

In our series the proliferation index increases from benign, to PIN, and cancer. The increase from benign to PIN and from benign to cancer is significant. If the proliferation rate as measured by Ki-67 is a predictor of the malignant potential of tissue, then PIN may be part of the way there.

Others smaller studies have supported this stepwise increase in MIB-1 expression when comparing benign tissue, with PIN and cancer [160;187;275;379;393].

Prostate cancer is an androgen responsive tumour, and by removing the stimulus for proliferation by chemical androgen ablation, the proliferation rate declines to a level significantly less than cancer, but still higher than benign tissue. This would support the view that androgens are not the only regulator of proliferation, and that these cells are displaying some autonomy from their endocrine regulators even at this early stage.

Bcl_2 staining increases from benign to malignant tissue, and so allowing the cancer cells to be resistant to intervention with androgen ablation or chemotherapy. Ki-67 increases from BPH to PIN and from BPH to cancer.

Implying that there is increased cell production in malignant and pre-malignant tissue compared to benign tissue.

Androgen ablation led to a decrease in Ki-67 staining, an increase in the number of specimens expressing bcl_2 and a decrease in the staining intensity for bax, implying LHRH not only leads to a decrease in the production of new cells but also to the selection of androgen resistant clones.

In summary in this thesis, it has been shown that the volume of cancer is proportio to the local stage of the disease; it is inversely proportional to the volume of beni and PIN tissue. The total gland volume is proportional to the volume of PIN. O gram of benign tissue correlates with 0.07 ng/ml of serum PSA, and one gram malignant tissue correlates with 0.8 ng/ml of serum PSA, the volume of PIN does r correlate with serum PSA.

With respect to the immunohistochemical studies, p53 is a rare event in localis disease; bcl_2 is significantly over expressed in PIN compared to cancer or beni tissue, and the intensity of bax staining significantly increases when compari benign tissue with PIN or malignant tissue, as does the expression of Ki-67.

Androgen ablation causes a significant increase in the expression of bcl_2, and decrea in the expression of Ki-67 and staining intensity of bax.

Chapter 9: Publications and Presentations.

9.1 Presentations resulting from this work.

CO-EXISTENCE OF INTRA-EPITHELIAL NEOPLASIA AND PROSTATE CARCINOMA.
A V Kaisary, **S S Sandhu**, P J Richmond and M Jarmulowicz.
Presented at the Sixth International Prostate Cancer Update, Denver Colarado USA, January 1996.

THE CORRELATION OF SYSTEMATIC SEXTANT BIOPSY WITH PATHOLOGICAL EXAMINATION OF RADICAL PROSTATECTOMY SPECIMENS.
S S Sandhu, VB Matveev, M Jarmulowicz, and A V Kaisary.
Presented at the British Association of Urological Surgeons, Annual Meeting June 1997.
The British Journal of Urology 1997:79(Suppl 4);abs104, p26-27.

THE EFFECT OF LHRH AGONISTS ON PROSTATIC INTRAEPITHELIAL NEOPLASIA.
M Jarmulowicz, **S S Sandhu**, T Walker and A V Kaisary.
Presented at the Pathological Society of Great Britain and Ireland, Winter Meeting January 1998.

THE CORRELATION OF SYSTEMATIC SEXTANT BIOPSY WITH PATHOLOGY.
S S Sandhu, M Jarmulowicz, T Walker, N Simmionds, Wilson C and A V Kaisary.
Presented at the Sixth Mediterranean Congress of Urology, Cairo, Egypt. Sept 1999.

THE CORRELATION OF PREOPERATIVE TRUS GUIDED SEXTANT BIOPSIES WITH RADICAL PROSTATECTOMY SPECIMENS.
S S Sandhu, T Walker, D St George, M Jarmulowicz, and A V Kaisary.
Presented at the Congress of the European Association of Urology, Feb 2002.
European Urology Suppliment Vol 1, No 1; abs659, p167.
Selected as the Best Poster in the session.

HIGH GRADE PROSTATIC INTRAEPITHELIAL NEOPLASIA, SERUM PSA, AND INVASIVE CANCER.
S S Sandhu, T Walker, D St George, M Jarmulowicz, and A V Kaisary.
Presented at the British Prostate Group Spring Meeting, April 2002.

PROSTATE CANCER, PROSTATIC INTRAEPITHELIAL NEOPLASIA AND SERUM PSA.
S S Sandhu, T Walker, C Thrasivoulou, M Jarmulowicz, and A V Kajsary.
Presented at Progress and Controversies in Oncological Urology (PACIO VII) and the seventh Congress of the Dutch Urological Association (DUA VII), October 2002.

TO INVESTIGATE USING MORPHOMETRIC CRITERIA THE RELATIONSHIP BETWEEN HIGH GRADE PROSTATIC INTRAEPITHELIAL NEOPLASIA PROSTATE CANCER, AND SERUM PSA.
S S Sandhu, T Walker, M Jarmulowicz and AV Kaisary.
Presented at the Société Internationale d'Urologie, Biannual Meeting October 2004. The British Journal of Urology International 2004: 94(Suppl 2);absUP-6.15, p194.

AN INVESTIGATION INTO THE RELATIONSHIP BETWEEN PROSTATIC INTRAEPITHELIAL NEOPLASIA AND PROSTATE CANCER.
S S Sandhu, T Walker, M Jarmulowicz and AV Kaisary.
Presented at the Société Internationale d'Urologie, Biannual Meeting October 2004. The British Journal of Urology International 2004: 94(Suppl 2);absUP-6.40, p200.

9.2 Publications resulting from this work.

TRIALS OF TREATMENT ARE NEEDED BEFORE TRIALS OF SCREENING.
S S Sandhu, R Morris, V Matveev, and A V Kaisary.
The British Medical Journal (1996): 312;709.

LOCALISED CARCINOMA OF THE PROSTATE: A PARADIGM OF UNCERTAINTY.
S S Sandhu and A V Kaisary.
Postgrad Med J 1997;**73:**691-696.

Appendix

Appendix 1: Area Calculation.

```
# Area measurements from micrographs for Sarb
# Chris Thras 4.01.2000
MSsetprop "FRAMEMODE",0
# showwindow "Editor",1

while 1
loops = 1 # reset loop number
   # imgsetpath "d:\sarb"
MSload "sarb"
# MSsetfeat "FIELDFEAT"


DBsetpath "c:\ks400\data\sarb"
dbnamef= "databases"
#specimen loop to allow data from several fields to be appended

while
read dbnamef,  "Input the field data base name you want to use"
n = DBexist (dbnamef)
if n == 0 : break
MBok "Database name exists - try again"
endwhile

while 1
imgdelete "*"
Gclear 0
imgnew 1,768,1024,1,"Grey"
imgdisplay 1
MBok " Copy and past total image from PSP into image 1"
imgnew 2,768,1024,1,"Grey"
MBok " Copy and past PIN image from PSP into image 2"
imgnew 3,768,1024,1,"Grey"
MBok " Copy and past CANCER image from PSP into image 3"
imgdisplay 1
greytran 1,4,"invers.col"
disaut 4,7,0,1
binfill 7,10
imgdisplay 2
greytran 2,5,"invers.col"
```

```
disaut 5,8,0,1
imgdisplay 3
greytran 3,6,"invers.col"
disaut 6,9,0,1

if loops == 1
imgdisplay 1
MSsetgeom
! MSmeasmask 10,1,dbnamef,0,0,10
imgdisplay 8
! MSmeasmask 8,1,dbnamef,1,0,10
imgdisplay 9
! MSmeasmask 9,1,dbnamef,1,0,10
datalist dbnamef,0,0

else

imgdisplay 1
MSsetgeom
! MSmeasmask 10,1,dbnamef,0,0,10
imgdisplay 8
! MSmeasmask 8,1,dbnamef,1,0,10
imgdisplay 9
! MSmeasmask 9,1,dbnamef,1,0,10
datalist dbnamef,0,0

endif
loops = loops + 1
# MByesno "Do you have another section in this group?"
   if _STATUS == 0 :break

endwhile
MByesno "Another sample/specimen?"
   if _STATUS == 0 :break
endwhile
beep 100,880

stop
```

Appendix No. 2 : Shrinkage

Pt Id	Fresh Wt	Fresh Vol	Fresh Density	Fx Wt	Fx Vol	Fixed Density	% Change in Wt	% Change in Vol
D	71.57	67.75	1.06	71.61	66.90	1.07	-0.05	1.25
G	61.20	56.70	1.08	60.90	55.80	1.09	0.49	1.59
H	44.01	43.60	1.01	42.86	41.61	1.03	2.61	4.57
M	42.70	40.97	1.04	41.96	39.65	1.06	1.73	3.21
To	60.12	57.24	1.05	59.70	56.72	1.05	0.69	0.91
Tu	70.79	69.95	1.01	70.30	66.45	1.06	0.69	5.00
Average	58.40	56.03	1.04	57.89	54.52	1.06	1.03	2.76

Therefore if the true volume is x, the volume measured should be multiplied by 100/(100-% Change in volume) ie.
1.03

Pt Id	Fx Slice Area	Mounted Slice Area H+E	Change in Area
D	1753.00	1560.62	10.97
G	1228.00	1027.30	16.34
H	1437.00	1165.50	18.89
M	1114.00	941.47	15.49
To	1842.50	1421.69	22.84
Tu	1535.60	1364.44	11.15
			15.95

Therefore if the fixed slice area is y, the area measured should be multiplied by 100/(100-% Change in area) ie.
1.19 and this will give the area of the H+E slides prior to being embedded in wax, and this should be multiplied by
1.03 and this will give the fresh area ie 1.22348

Appendix 3: Slice thickness

Pt Id	Slice 2	Slice 3	Slice 4	Slice 5	Slice 6	Slice 7	Slice 8	Total Slice Thickness	Av Slice thickness
D	3.17	3.38	3.39	3.31	3.52	3.3	3.77	23.84	3.41
G	2.77	2.92	3.08	3.01	3.06	2.7	3.62	21.16	3.02
H	3.02	3.36	3.32	3.42	3.75	4.28	3.28	24.43	3.49
M	3.19	3.26	3.41	3.79	3.61	4.21	4.33	25.80	3.69
To	2.42	2.63	3.04	3.38	3.58	3.23	3.29	21.57	3.08
Tu	3.88	3.62	3.19	3.24	3.36	3.41	3.63	24.33	3.48

To get the volume one should multiply the area by the thickness of the slices multiplied by the shrinkage fraction for the fixation in formalin i.e. 1.028335. i.e. 3.46. This has been corrected to one decimal place as 3.5mm.

Appendix 4:
Preparation of 3-Aminopropyltriethoxysilane (APES) coated slides.
Royal Free Hospital School of Medicine – Standard Operating Procedure No.5

METHOD (van Prooijen-Knegt ACet al. 1983; MaddoxPH and Jenkins, 1987).

1. Degrease slides in hot detergent solution, rinse in distilled water then alcohol (740 P) and air dry for 1 0 minutes.
2. Immerse slides in a freshly prepared 2 % solution of APES in acetone- minimum 1 minute.
3. Drain slides briefly and wash in distilled water – 2 quick dips.
4. Drain slides on paper towels and dry at 40^0C over night in oven on paper towels.
5. Store at room temperature in empty slide boxes.

Appendix No.5
Preparation Paraffin Sections for immunohistochemistry
Royal Free Hospital School of Medicine – Standard Operating Procedure No.2

1. Cool paraffin blocks.
2. Cut paraffin sections at 3 micro m.
3. Float sections on 20% alcohol then 45^0C water bath.
4. Pick up sections on APES coated glass slides as appropriate.
5. Place slides in 37^0C oven over night
6. De-parafinise sections and take them to alcohol.

a)	Xylene 1	5 minutes
b)	Xylene 2	5 minutes
c)	Xylene 3	5 minutes
d)	Absolute Alcohol	2 minutes
e)	Absolute Alcohol	2 minutes
f)	Absolute Alcohol	2 minutes
g)	Double distilled water	2 minutes

Appendix No.6: Staining procedure.
Royal Free Hospital School of Medicine – Standard Operating Procedure No.13

1. Deparafinise sections and take to alcohol.
2. Transfer sections to humidity chamber and block endogenous peroxidase activity.
3. Wash in 3 times changes of de-ionised or distilled water for 2 minutes each.
4. Use antigen retrieval system.
5. Transfer slides to a humidity chamber and cover sections with two changes of Tris buffered saline (TBS) for 5 minutes each.
6. Block non specific binding by covering with normal goat serum 1/10 in TBS for 15 minutes at room temperature.
7. Drain and cover sections with optimally diluted primary antibody diluted in TBS. Leave sections in humidity chamber for the appropriate time and temperature for the antibody.
8. Rinse in TBS twice for 3 minutes.
9. Drain and cover sections with biotinylated goat anti rabbit/mouse antisera 1/200 in 4% normal human sera for 30 minutes in a humidity chamber at room temperature.
10. Repeat step 8.
11. Drain and cover sections with STR-ABC 1/200 for 30 minutes in a humidity chamber.
12. Repeat step 8.
13. Develop sections with freshly prepared Diaminobenzidine (DAB) solution.
14. Wash in distilled water for 5-10 minutes.
15. Counter stain with Mayer's haemotoxylin for 2 minutes.
16. Rinse in distilled water. Differentiate in 0.5% acid alcohol and blue for 5 minutes.
17. Dehydrate, clear, and mount.
18. Sites of immunoperoxidase activity = Brown,
    Nuclei = Blue.

Appendix No. 7 : Blocking endogenous peroxidase activity and alkaline phosphatase activity.
Royal Free Hospital School of Medicine – Standard Operating Procedure No.3

Hydrogen Peroxide/Water Method

1. De-paraffinise sections and take to water
2. Transfer slides to a humidity chamber at room temperature and cover the sections with freshly prepared 0. 5 % H_2O_2 in distilled water for 10-15 minutes. This solution contains: 0.2 mls H_2O_2 (30% W/Vol.) and 11.8 mls water (enough for approx, 20 slides).
3. Wash sections in distilled water 3 times and proceed with immunostaining method.

Appendix No. 8
Buffers for Immuno-histochemistry

EQUIPMENT
Magnetic stirrer
pH meter
Balance
Conical flask
1 0 Litre plastic vat
Magnetic flea

A. Tris buffered saline (TBS) pH 7.6.
REAGENTS

Sodium chloride	80 gm
Tris (Tris hydroxymethylamine)	6.6 gm
1 M Hydrochloric Acid	44 mls
Double distilled water	10 litres

METHODS

1. Mix reagents with 2 litres of distilled water on a magnetic stirrer with a magnetic flea.
2. When components are dissolved transfer solution to a plastic 10-litre vat.
3. Add 8 litres of water and mix thoroughly.
4. Take an aliquot and pH to 7.6 (+/-0.05) using buffer components.
5. Buffers must be as fresh as possible in order to prevent adverse affects on staining. If storing, keep at 4 'C ready for use.

B. Phosphate.buffered saline (PBS) pH 7.4

REAGENTS

PBS tablets (Oxoid)	1
Double distilled water	100mls

METHODS

1.Mix reagents and check pH is 7.4 (+/- 0.05). If not correct ph check water pH and/or quality of tablets.

Appendix No. 9
Microwave technique for immuno-histochemistry [355 352 354 353 383 384]
EQUIPMENT
pH meter
Microwave (700W) oven with rotation plate.
Balance
Plastic coplin jars
1 L conical flask

Citric acid buffer
REAGENTS

Citric Acid Monohydrate	2.1g
Double distilled water.	10 litres
2M NAOH	13 mls

pH to 6.0 with buffer components

METHOD
1. De-paraffinise sections (appendix 2) sections must be mounted on APES slides (see appendix 1) or there will be section loss.
2. Block endogenous peroxidase activity (appendix 4)
3. Wash slides with PB S.
4. Fill plastic coplin jar 4/5 with citric acid buffer 0.01 m Citric acid buffer solution and submerge sections.

Appendix 10 Raw Morphimetric data

Pt Id	PSA	Total vol	Ca Vol	PIN Vol	Benign vol	Pros Weight
1	7	39605.6	2467.9	544.6	36593.1	42.03
2	5	37719.8	1922.2	250.9	35546.8	50.70
3	13.5	80401.6	8947.7	44.6	71409.3	93.00
4	14	64846.7	2656.1	307.5	61883.1	75.00
5	4.5	42495.4	2984.7	875.4	38635.3	52.30
6	9.6	59843.0	901.5	45.7	58895.7	67.80
7	9.6	42027.8	3774.7	836.9	37416.2	57.00
8	9.2	60964.2	612.5	243.0	60108.7	79.00
9	4.9	67633.6	8.9	12.5	67612.2	67.00
10	8.4	67063.1	22.2	1157.2	65883.7	77.80
11	5.6	80449.2	5089.8	234.5	75124.8	90.00
12	7.2	60497.7	2243.0	2300.2	55954.4	74.20
13	10	57438.6	957.8	117.9	56362.9	70.00
14	6.1	35346.4	1965.9	117.3	33263.2	47.60
15	9.7	89791.2	130.8	1132.1	88528.3	91.30
16	5.9	37108.8	3632.7	153.0	33323.1	44.00
17	5.5	70380.8	652.7	775.1	68952.9	75.50
18	6.2	39543.2	3265.3	487.2	35790.8	55.00
19	6.9	31638.0	1880.8	278.5	29478.7	46.00
20	5	40453.1	1543.9	510.0	38399.3	54.00
21	5.4	56137.8	430.2	462.9	55244.7	59.80
22	13.7	74299.1	2024.0	2728.5	69546.5	82.70
23	6.1	36654.9	1793.4	397.5	34464.0	42.00
24	13.2	39420.7	857.8	1514.2	37048.6	41.86
25	32.4	62789.4	15150.6	249.9	47388.9	88.40
26	5.3	41864.9	2519.3	180.8	39164.8	50.00
27	16.1	64089.7	293.3	479.5	63316.8	63.29
28	8.1	84674.2	4073.2	465.4	80135.7	99.00
29	10.3	55855.5	113.5	220.4	55521.6	71.00
30	9.6	83056.3	3704.6	400.0	78951.8	89.00
31	7.5	40394.0	1507.8	509.3	38376.9	43.01
32	4.1	46648.7	382.6	411.0	45855.1	57.80
33	5.2	29175.3	2767.0	64.9	26343.4	35.00
34	3.6	24503.2	1934.3	78.6	22490.3	32.20
35	5.7	69594.5	5324.7	343.3	63926.5	87.50
36	8.3	50470.0	3074.4	0.0	47395.6	59.00
37	6.2	28633.4	1564.1	283.5	26785.8	39.50
38	10.1	40458.6	1254.1	266.7	38937.8	48.00
39	11	39406.3	656.2	29.7	38720.4	61.60
40	7.3	43101.7	903.4	296.2	41902.1	52.30
41	5	62086.4	393.9	70.4	61622.1	81.00
42	15	23074.0	1427.0	132.2	21514.7	37.00
43	7.9	37088.2	1638.8	174.2	35275.2	42.40
44	12.4	34313.5	2153.4	207.1	31953.0	51.10
45	7.2	72421.6	694.0	1558.4	70169.2	67.80
46	8.2	70390.9	664.0	358.6	69368.3	71.00

47	4.2	19359.9	5839.4	222.6	13298.0	31.80
48	3	71721.5	139.1	985.9	70596.5	79.00
49	11.5	68491.4	3170.7	392.2	64928.5	73.80
50	7.4	41647.8	8847.8	328.5	32471.5	59.10
51	3.6	43831.8	946.1	2259.3	40626.3	54.00
52	10.7	51745.4	209.9	2406.5	49129.1	67.00
53	12.6	30462.3	2778.9	32.9	27650.5	42.00
54	7	34510.7	520.9	887.3	33102.4	52.00
55	5.3	32558.9	1874.2	605.9	30078.8	44.00
56	12.1	52898.0	12286.9	438.5	40172.5	57.35
57	5.3	35251.5	4213.2	156.8	30881.5	46.50
58	7.1	38953.1	3436.1	152.0	35365.0	42.00
59	8.1	67732.9	160.6	354.4	67217.9	49.75
60	8.3	45525.4	1550.7	1535.8	42438.9	65.00
61	45	59354.2	4282.1	635.8	54436.2	62.90
62	6.2	60233.1	854.2	255.9	59123.0	74.60
63	9.9	32028.5	5823.5	572.9	25632.1	34.67
64	14.4	31259.8	4905.4	449.5	25904.9	43.00
65	6.3	48891.0	57.2	23.5	48810.3	59.00
66	6	35443.7	2725.4	304.1	32414.2	41.00
67	11.5	56645.0	761.9	933.8	54949.3	70.00
68	14	51785.8	2094.0	21.6	49670.3	68.40
69	4.8	29300.4	1186.6	18.4	28095.4	39.00
70	9	24580.4	5062.5	26.5	19491.4	26.00
71	13	155519.0	142.2	1939.8	153437.0	173.00
72	6.2	47481.5	665.3	125.1	46691.1	55.70
73	6.7	34677.6	3283.6	378.7	31015.2	43.50
74	6.2	41129.4	2284.5	400.7	38444.3	48.22
75	7.5	48382.7	507.7	1151.6	46723.4	58.00
76	13.4	64003.2	466.7	2.9	63533.6	68.60

Appendix 11: Raw Immunohistochemical data.

Data obtained from regions of prostate cancer.

	Number of + cells P53 Ca	P53 Ca	PSA Ca	Number of + cells Bcl2 Ca	BCl2 Ca	Ki67 Ca	Bax Ca
1	0	0	2	0	0	1	1
2	0	0	2	0	0	1	1
3	0	0	3	0	0	1	2
4	0	0	3	0	0	1	0
5	0	0	2	60	1	1	2
6	0	0	3	0	0	1	3
7	100	2	1	40	1	1	1
8	0	0	2	0	0	1	0
9	0	0	3	50	1	1	3
10	0	0	1	0	0	1	2
11	0	0	2	0	0	1	3
12	0	0	2	0	0	1	1
13	0	0	3	0	0	1	2
14	0	0	2	10	1	1	2
15	100	2	3	100	2	1	3
16	0	0	3	0	0	1	3
17	0	0	3	0	0	1	1
18	0	0	3	20	1	1	3
19	0	0	2	10	1	1	2
20	0	0	2	0	0	1	2
21	0	0	2	0	0	1	1
22	100	1	2	0	0	1	1
23	0	0	1	0	0	1	1
24	0	0	2	80	2	1	2
25	0	0	3	0	0	35	1
26	0	0	3	0	0	8	1
27	0	0	3	0	0	5	2
28	0	0	3	0	0	4	2
29	0	0	2	0	0	10	2
30	0	0	2	0	0	3	1
31	0	0	2	0	0	3	2
32	0	0	3	100	1	5	1
33	0	0	2	0	0	1	2
34	0	0	3	0	0	1	2
35	50	1	1	50	1	7	3
36	0	0	1	0	0	0	2
37	80	2	1	0	0	7	2
38	0	0	3	0	0	10	2
39	0	0	2	0	0	3	1
40	0	0	2	0	0	2	2
41	0	0	3	0	0	3	3
42	0	0	2	0	0	5	2
43	0	0	1	0	0	3	2
44	0	0	2	0	0	10	2

45	0	0	3	0	0	1	1
46	0	0	3	50	1	10	2
47	0	0	3	30	1	3	2
48	0	0	2	0	0	15	2
49	0	0	2	0	0	5	2
50	0	0	3	0	0	5	1
51	0	0	3	40	1	10	2
52	0	0	1	0	0	15	2
53	0	0	3	0	0	1	1
54	0	0	2	0	0	12	3
55	0	0	2	0	0	1	1
56	0	0	2	10	1	30	1
57	0	0	3	0	0	20	3
58	0	0	1	100	1	10	2
59	10	1	2	0	0	5	1
60	0	0	2	0	0	6	1
61	0	0	2	0	0	10	2
62	0	0	1	50	1	4	2
63	0	0	1	0	0	8	1
64	50	1	3	50	2	1	1
65	0	0	1	30	1	20	2
66	50	2	2	0	0	5	2
67	0	0	3	0	0	1	1
68	0	0	1	10	1	5	2
69	0	0	2	80	1	5	2
70	0	0	3	0	0	10	2
71	0	0	3	100	1	5	2
72	0	0	0	0	0	0	1
73	100	1	3	50	1	1	3
74	0	0	0	100	2	3	1
75	0	0	2	0	0	10	2
76	0	0	1	0	0	5	2
77	0	0	2	0	0	10	3
78	0	0	1	50	1	5	2
79	0	0	3	0	0	1	1
80	0	0	2	60	1	5	2
81	0	0	2	100	1	15	3
82	0	0	3	0	0	7	2
83	0	0	2	0	0	3	2
84	0	0	2	0	0	1	2
85	0	0	1	0	0	10	1
86	0	0	3	10	1	10	3
87	0	0	1	0	0	4	2
88	0	0	2	0	0	10	2
89	0	0	2	0	0	15	1
90	0	0	3	0	0	1	1
91	0	0	2	0	0	20	2
92	0	0	2	0	0	10	1

Data obtained from regions of PIN.

	Number of + cells P53 PIN	P53 PIN	PSA PIN	Number of + cells Bcl2 PIN	BCl2 PIN	Ki67 PIN	Bax PIN
1	0	0	3	60	2	1	1
2	0	0	2	0	0	1	0
3	0	0	3	80	1	1	1
4	0	0	3	0	0	1	2
5	0	0	3	0	0	1	2
6	0	0	3	60	2	1	2
7	0	0	3	20	1	1	1
8	0	0	3	100	2	1	1
9	0	0	2	80	2	1	1
10	0	0	3	100	2	1	1
11	0	0	3	50	2	1	1
12	0	0	3	30	2	1	2
13	0	0	3	25	2	1	1
14	0	0	3	40	1	1	2
15	0	0	3	100	2	1	1
16	0	0	3	100	2	1	1
17	100	3	3	10	2	1	1
18	0	0	3	80	2	1	2
19	0	0	3	60	2	1	3
20	0	0	3	5	1	1	2
21	0	0	3	25	2	1	2
22	0	0	2	0	0	1	2
23	0	0	3	0	0	0	2
24	0	0	2	60	1	5	2
25	0	0	2	100	1	1	2
26	25	2	3	80	2	5	1
27	0	0	2	60	2	2	3
28	0	0	3	0	0	5	2
29	0	0	2	20	1	5	2
30	0	0	2	100	1	3	2
31	0	0	2	0	3	5	2
32	0	0	3	10	2	5	2
33	0	0	3	10	2	1	2
34	0	0	3	60	1	2	1
35	50	2	3	100	1	5	3
36	0	0	2	100	5	4	2
37	50	1	2	0	0	7	2
38	0	0	3	30	1	5	2
39	0	0	2	70	3	5	1
40	0	0	3	60	2	4	2
41	0	0	3	50	2	3	3
42	0	0	1	80	2	1	1
43	0	0	1	0	0	3	2
44	90	1	3	50	1	3	2
45	0	0	3	100	2	2	2
46	0	0	3	30	2	3	2

47	0	0	3	80	2	20	2
48	0	0	2	0	0	15	2
49	0	0	2	70	2	2	2
50	0	0	3	70	2	1	2
51	0	0	3	0	0	5	2
52	0	0	2	30	2	3	2
53	0	0	3	10	1	1	1
54	0	0	2	30	2	5	3
55	0	0	3	100	2	1	1
56	0	0	2	5	1	40	1
57	0	0	3	30	1	3	3
58	0	0	2	80	2	5	2
59	0	0	2	0	0	5	0
60	0	0	0	0	0	0	0
61	0	0	3	50	1	15	2
62	0	0	3	50	2	1	2
63	0	0	2	40	2	5	1
64	0	0	0	0	0	0	0
65	0	0	1	10	2	15	2
66	0	0	3	100	2	10	2
67	0	0	3	0	0	1	1
68	0	0	2	10	2	2	2
69	0	0	3	100	1	8	2
70	0	0	3	0	0	10	2
71	0	0	3	70	1	5	0
72	0	0	3	10	1	5	2
73	0	0	2	100	2	1	0
74	0	0	0	0	0	0	1
75	0	0	3	0	0	10	2
76	0	0	2	80	1	1	2
77	0	0	3	30	1	25	3
78	0	0	3	30	1	3	2
79	0	0	1	100	3	1	1
80	0	0	3	50	2	5	2
81	0	0	2	100	1	15	3
82	0	0	3	0	0	5	2
83	0	0	3	60	2	3	2
84	0	0	3	10	1	1	2
85	0	0	2	50	1	2	0
86	0	0	3	60	2	3	3
87	0	0	3	100	1	2	2
88	0	0	2	100	2	5	2
89	0	0	2	40	1	5	1
90	0	0	2	80	1	1	1
91	0	0	2	30	1	20	2
92	0	0	3	100	1	10	1

Data obtained from specimens with 3 months of androgen ablation.

	Number of + cells	P53 LHRH	Number of + cells	BCl2 LHRH	Ki67 LHRH	Bax LHRH
1	0	0	100	3	1	1
2	0	0	70	1	1	1
3	0	0	0	0	1	1
4	0	0	50	1	1	1
5	0	0	100	2	1	2
6	0	0	100	2	1	2
7	0	0	0	0	25	1
8	0	0	60	1	1	2
9	50	3	0	0	1	2
10	80	1	100	2	1	1
11	0	0	0	0	2	2
12	100	1	0	0	1	1
13	0	0	100	1	1	2
14	80	1	100	1	1	1

Data obtained from benign (control specimens)

	P53 Benign	PSA Benign	Number of + cells	BCl2 Benign	Ki67	Bax
1	0	3	0	0	0	1
2	0	3	0	0	0	2
3	0	3	0	0	0	1
4	0	3	0	0	0	2
5	0	3	0	0	0	1
6	0	3	0	0	0	2
7	0	3	0	0	0	0
8	0	3	0	0	0	2
9	0	3	0	0	0	2
10	0	3	0	0	0	1
11	0	3	0	0	0	2
12	0	3	0	0	0	1
13	0	3	0	0	0	1
14	0	3	0	0	0	2
15	0	3	0	0	0	1
16	0	3	0	0	0	2
17	0	3	0	0	0	1
18	0	3	0	0	0	1
19	0	3	0	0	0	1

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